Promoting Recovery After Stroke: Experimental Studies To Understand Key Mechanisms Of Rehabilitation And Neuronal Plasticity

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Zusammenfassung

Schlaganfall ist eine der Hauptursachen für erworbene Behinderungen im Erwachsenenalter weltweit und die aktuell verfügbaren therapeutischen Möglichkeiten sind begrenzt. Das liegt unter anderem daran, dass wir immer noch nur unzureichend die Pathologie des Schlaganfalls verstehen, insbesondere Reorganisations- und Regenerationsprozesse, die im Gehirn nach dem Insult stattfinden um funktionelle Erholung zu ermöglichen. Gerade unser Wissen um das Zusammenspiel von intrinsischen Wachstumsprozessen nach Schlaganfall und externen therapeutische Interventionen, wie z.B. Rehabilitationstraining, Elektrostimulation (TMS) oder der Gabe von Wachstums fördernden Substanzen, ist immer noch sehr lückenhaft.
Die hier vorgelegte Arbeit beschäftigt sich mit der Untersuchung von intrinsisch plastischen Mechanismen nach Schlaganfall, um neue Möglichkeiten für das Design von optimierten Rehabilitationstherapien zu finden, die direkt positiv auf interne Wachstumsmechanismen einwirken und damit ein grösseres Potential für die Wiederherstellung von verlorenen, insbesondere motorischen Funktionen eröffnen können.
Im **ersten Kapitel** fassen wir das aktuelle Verständnis von Mechanismen, die an spontanen Erholungsprozessen beteiligt sind, zusammen. Wir präsentieren Beispiele für experimentelle Interventionen, die die Wachstumsprozesse im Gehirn nach Schlaganfall beschleunigen könnten, wie z.B. eine Antikörpertherapie gegen den stärksten Wachstumshemmer im zentralen Nervensystem, das Nogo-A Protein. Weil es Hinweise für ein kritisches Zeitfenster für Wachstum und die Anwendung von Rehabilitationstraining gibt, diskutieren wir verschiedene Ansätze für kombinierte Therapieoptionen.
Im **zweiten Kapitel** legen wir mögliche methodische Hindernisse in der aktuellen experimentellen Schlaganfallforschung dar: Bisherige Studien waren im Hinblick auf das Verständnis des Zusammenhangs von Reorganisationsprozessen nach Schlaganfall und dem Grad der funktionellen Erholung vor allem korrelativ. Deshalb stellen wir neue Technologien vor, die in den letzten Jahren im Bereich der Neurowissenschaften entwickelt wurden und die der Schlaganfallforschung neue Möglichkeiten eröffnen könnten, um kortikale Reorganisation zu studieren, neuronale Schaltkreise zu manipulieren und die zu Grunde liegenden molekularen Mechanismen zu verstehen.
Kapitel 3 fasst unsere Ergebnisse über Reorganisationsprozesse in der Region um den Schlaganfall, der sogenannten Penumbra, zusammen, die wir nach einem kleinen Schlaganfall im Mausmodell, der das kortikale Hirnareal für die Handfunktion zerstört hatte, beobachtet haben. Wir haben in einer Kombination von Licht-basierter kortikaler Stimulation zur Kartierung der funktionellen Hirnareale für die Vorder-und Hinterpfote und detaillierter kinematischer Analyse festgestellt, wie wichtig die Reorganisation innerhalb der Penumbra ist, um die durch den Schlaganfall zerstörte Greiffunktion wiederherzustellen: In der Penumbra entsteht ein neues Zentrum der Handrepräsentation, die auch das Motor-Engramm für die Greiffunktion enthält.

Das spontane Erholungspotenzial nach einem grossen Schlaganfall ist begrenzt. Im **Kapitel 4** haben wir vier verschiedene Rehabilitationsparadigmen in einem Rattenmodell nach einem grossen photothrombotischen Schlaganfall im sensomotorischen Cortex angewendet. Wir haben dabei herausgefunden, dass der richtige Zeitpunkt entscheidend ist für den Grad der funk-

tionellen Erholung, wenn zwei mögliche Therapieoptionen kombiniert werden: Ratten, die zunächst für zwei Wochen eine Immuntherapy mit einem Antikörper gegen Nogo-A erhielten und nachfolgend für zwei Wochen intensives Training der Handfunktion, erholten sich fast vollständig, im Gegensatz zu Ratten, die ihr Training parallel zur Antikörpertherapie innerhalb der ersten zwei Wochen nach Schlaganfall durchführten. Diese zeigten kaum eine Erholung der Greiffunktion und waren damit schlechter als die Kontrolltiere. Anatomisch spiegelte sich der unterschiedliche Grand der Erholung in komplett verschiedenen Wachstumsmustern von neu aussprossenden corticospinalen Fasern wieder, die ihren Ursprung von Neuronen in der gesunden Hemisphäre nahmen. Während corticospinale Fasern von Tieren mit guter Erholung ein normales Verzweigungmuster aufwiesen und vorwiegend im motorischen Vorderhorn der grauen Substanz endeten, war das Faserwachstum von Tieren mit schlechter Erholung der Greiffunktion, die parallel zur Immuntherapie trainiert worden waren, überschiessend, mit einer Ueberzahl an Synapsen, wobei die Fasern zum Teil falsche Regionen des Rückenmarks ansteuerten. Als wir in den gut erholten, d.h. nach Antikörpertherapie trainierten Ratten, temporär corticospinale Fasern aus einer bestimmten Region im pre- und primärmotorischen Cortex der gesunden Hemisphäre abschalteten, die auf Rückenmarksebene über die Mittellinie von der gesunde auf die kranke Rückenmarksseite hinüberwuchsen, war die wiedergewonnene Handfunktion der Ratten erneut verloren. Damit zeigte sich diese Gruppe von im Rückenmark die Mittellinie kreuzenden corticospinalen Fasern als entscheidend für die Wiederherstellung der Feinmotorik in der Vorderpfote nach Schlaganfall.
Ein Kandidaten-basiertes Screening gab erste Hinweise (wie in **Kapitel 5** beschrieben), dass verschiedene Rehabilitationsparadigmen definierte örtliche und zeitliche Expressionsmuster von Wachstums fördernden Faktoren und Neurotrophinen einleiten. Insbesondere die erhöhte Expression des Brain-derived neurotrophic factor *(BDNF)* Gens in der denervierten Seite des zervikalen Rückenmarks war mit einer guten Erholung der motorischen Funktion in den Tieren korreliert. Im Gegensatz dazu waren Anzeichen von Gliose und überschiessende Synapsenbildung die vorrangigen Merkmale im Gehirn und Rückenmark von Tieren mit schlechter Erholung, die gleichzeitig zur Anti-Nogo A Antikörpertherapie Rehabilitationstraining erhalten hatten.

Diese Arbeit gibt Hinweise darauf, dass genau orchestrierte, akkurat durchgeführte, zeitabhängige interne Reparaturmechanismen die neuronale Reorganisation als Grundlage für die Wiederherstellung verlorener Funktionen kontrollieren. Weiterhin zeigen wir, wie wichtig es für die funktionelle Erholung ist, sorgfältig über die Konzeption von Rehabilitationsschemata nach Schlaganfall nachzudenken, insbesondere, wenn verschiedene Therapieoptionen kombiniert werden, wie z.B. Trainung und Substanzen, die das Nervenfaserwachstum fördern. Ein Rehabilitationsansatz hat dann vor allem Erfolg, wenn er in einem ersten Schritt zunächst intrinsische Wachstumsprozesse weiter unterhält und ankurbelt (z.B. durch die Gabe von Anti-Nogo A Antikörpern) und Training in einem zweiten Schritt erfolgt, das die Stabilisierung von bedeutsamen neuen Nervenschaltkreisen unterstützt, während funktionslose Schaltkreise verschwinden. Im Gegensatz dazu können Rehabilitationsansätze nicht förderlich sein für die Wiedererlangung und Erholung von verlorenen Funktionen, wenn der Rehabilitationsansatz zum falschen Zeitpunkt oder in zu hoher Intensität erfolgt. Gerade initial nach Schlaganfall scheint das Gehirn in einer besonders sensitiven Phase zu sein. Zu intensive, erzwungene Rehabilitationsmassnahmen sollten in dieser Phase, in der erhöhte Umbauvorgänge im Gehirn stattfinden, nur mit grösster Vorsicht angegangen werden.

Summary

Stroke is a major cause of adult disability worldwide and the treatment options available are limited so far - in part, because we are still lacking detailed knowledge about stroke pathology and reorganization processes taking part in the brain after the ischemic insult to enable the recovery of lost function. In particular, we do not understand the interplay between intrinsic growth-promoting processes induced by stroke and external therapeutical interventions such as rehabilitative training, electrical stimulation or plasticity-boosting therapies.
The present work aims at understanding intrisic plastic mechanisms after stroke in order to optimize the design of rehabilitation schedules which enhance these plastic processes and thus provide restoration of lost function.
In **chapter 1** we provide a general overview concidering the current understanding of mechanisms involved in spontaneous recovery after stroke. Furthermore we introduce experimental interventions to enhance growth and plasticity, with an emphasis on Anti-Nogo A immunotherapy. As there is evidence for a critical window of growth and rehabilitative training, we discuss different approaches of combinatorial rehabilitative schedules.
In **chapter 2** we highlight the caveats of current experimental stroke research emphasizing that our understanding of rewiring processes after stroke in relation to functional outcome levels have been merely correlative so far. We therefore review new technologies recently developed for neuroscientific applications which are also powerful tools to study cortical reorganization, to manipulate rewiring neuronal circuits and to identify the underlying molecular profiles.
Chapter 3 summarizes our results of examining reorganization in the peri-infarct area after a small focal stroke destroying the mouse forelimb motor cortex. Here we used a combination of light-based motor mapping measuring cortical map shifts and detailed kinematic analysis to reveal the functional relevance of the peri-infarct cortex for the organization of a new center for forelimb function and the re-establishment of motor engrams lost due to the stroke.
Spontaneous recovery after large strokes is very limited. In **chapter 4** we thus applied four different rehabilitation paradigms in a rat model for large photothrombotic strokes targeting the sensorimotor cortex. We found that timing is crucial when two treatment strategies are combined: Rats receiving first Anti-Nogo A immunotherapy for two weeks after stroke followed by two weeks of intensive rehabilitative training of the forelimb showed almost full restoration of lost function while simultaneous training to Anti-Nogo-A immunotherapy resulted in very poor outcome levels, which were found to be even below control groups. Anatomically these distinct outcome levels were reflected by distinct growth patterns of newly out-sprouting corticospinal fibers originating in the healthy contralesional hemisphere. While in the well recovered group of rats, these fibers formed a near normal branching and termination pattern in the ventral horn of the spinal cord, aberrant branching and too high numbers of synapses, in part in wrong areas of the spinal cord were apparent in rats with poor functional recovery having received simultaneous training with Anti-Nogo A immunotherapy. Temporarily silencing corticospinal fibers which originated from a distinct area within the premotor and rostral motor cortex of the healthy hemisphere, crossed the midline at the level of the cervical spinal cord and targeted the denervated grey matter, resulted in a decline of regained forelimb function in rats with good functional outcome (after sequential training application), thus demonstrating

the crucial relevance of these newly outsprouting fibers for the restoration of skilled motor function. A candidate-based screening provided first insights (as described in **Chapter 5**) that different rehabilitation paradigms induce distinct spatio-temporal expression patterns of growth-promoting genes and neurotrophic factors. Induction of the brain-derived neurotrophic factor (*BDNF*) gene in particular in the denervated cervical hemi-spinal cord was associated with good functional outcome. In contrast, signs for gliosis and extensive synpatogenesis were predominant characteristics in the brains and spinal cords of animals, which had concurrent training to Anti-Nogo A antibody application.

In summary, this work provides evidence for precisely orchestrated time-dependent internal repair processes controlling mechanisms of neuronal rewiring for the restoration of lost motor functions after stroke. Furthermore we show here the importance of carefully designing rehabilitation schedules after stroke, in particular if different therapeutic options such as training and growth enhancing agents are combined: A rehabilitative approach which first supports and enhances the intrinsic potential of the brain and spinal cord to reinforce lost functions by boosting the growth of new fibers (e.g. through growth-promoting therapies such as Anti-Nogo A antibody application) followed by a phase of stabilization of the meaningful newly formed circuits and pruning of non-functional ones through training, may result in robustly enhanced recovery of motor function. In contrast, rehabilitative schedules may become less beneficial if applied at the wrong time and intensity. In particular in the early phase after the injury, the brain seems to be in a delicate vulnerable state. Forced rehabilitative therapies should therefore be initiated with great caution during this period of circuit plasticity.

Contents

Part I

Introduction

Chapter 1

Finding an optimal rehabilitation paradigm after stroke: enhancing fiber growth and training of the brain at the right moment

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Abstract

After stroke the central nervous system reveals a spectrum of intrinsic capacities to react as a highly dynamic system which can change the properties of its circuits, form new contacts, erase others, and remap related cortical and spinal cord regions. This plasticity can lead to a surprising degree of spontaneous recovery. It includes the activation of neuronal molecular mechanisms of growth and of extrinsic growth promoting factors and guidance signals in the tissue. Rehabilitative training and pharmacological interventions may modify and boost these neuronal processes, but almost nothing is known on the optimal timing of the different processes and therapeutic interventions and on their detailed interactions. Finding optimal rehabilitation paradigms requires an optimal orchestration of the internal processes of re-organization and the therapeutic interventions in accordance with defined plastic time windows.

In this review we summarize the mechanisms of spontaneous plasticity after stroke and experimental interventions to enhance growth and plasticity, with an emphasis on anti-Nogo-A immunotherapy. We highlight critical time windows of growth and of rehabilitative training and consider different approaches of combinatorial rehabilitative schedules. Finally, we discuss potential future strategies for designing repair and rehabilitation paradigms by introducing a '3 step model': determination of the metabolic and plastic status of the brain, pharmacological enhancement of its plastic mechanisms, and stabilization of newly formed functional connections by rehabilitative training.

The human brain works wonders to fulfill the requirements of every-day life. These unique capacities are then fully esteemed when all of a sudden even simple activities fail or become a problem: Cerebral strokes leave the victims with often large psychical and physical impairments - from vision problems to aphasia and motor deficits – leading to the number one cause of adult disability worldwide with great impact on public health. In the acute phase, 'time is brain' – ruptured blood vessels (hemorrhagic stroke) or aggregates of platelets and blood cells that clog cerebral blood vessels (ischemic stroke) cause acute shortage of glucose and oxygen resulting in metabolic distress and long-term neuronal cell loss. The destruction process is complex and can only be dampened in the case of the ischemic stroke by very early intervention (within 4-6 hrs) with thrombolysis, (Hacke et al. 2008). Currently, only about 10% of all stroke patients reach a hospital early enough or fulfill the criteria for being able to receive thrombolysis in the therapeutic time window. Prognosis and recovery then depend on the location and extent of the stroke lesion.Clinically, the most successful therapy to further enhance this recovery of function is rehabilitative training. Rehabilitation as a term 'to reach and maintain optimal functioning in physical, intellectual, psychological and/or social domains'(WHO. International classification of functioning disability Health ICF. Geneva: WHO; 2001) is evidence based medicine and does not exclude a specific subgroup of patients.

Nevertheless, for many rehabilitative interventions, in particular those for long-term or chronic rehabilitation, robust data or adequately controlled studies are lacking (Quinn et al., 2009): E.g. comparisons between different training methods in current use could not show that any particular physiotherapy or stroke rehabilitation strategy is superior to another (Johansson et al., 2000).
Consequently optimal rehabilitation strategies can only be defined if we understand the way in which training and the rehabilitation protocol influences the neurobiology of the central nervous system with priority on the aspects of timing, kind and intensity of rehabilitative training. Measurable endpoint criteria for rehabilitative outcome are required in order to achieve two purposes: The adjustment of the ideal rehabilitative strategy to the individual patient, and the choice of the optimal therapy protocol.

In this review we focus on mechanisms of spontaneous recovery after stroke, on rehabilitative designs to enhance plasticity, on growth promoting mechanisms with an emphasis on Anti-Nogo A immunotherapy, and on the time windows of rehabilitative training and pharmacological interventions and the combination of both.

1.1 Mechanisms of spontaneous recovery after stroke – from human patients to animal models

For many years people have thought that the hardware of the brain is that 'hard', that once an incident such as stroke happens, brain areas and functions are lost forever. The old paradigm of the adult CNS as a stable and static structure, consisting of billions of nerve cells and circuits, has now been replaced by a much more dynamic view of the CNS which includes processes of growth, connectivity changes and areal remodeling that can occur after CNS injury or stroke and plays an important role in recovery and functional repair.
Spontaneous recovery is seen in stroke patients weeks to months after the incident. However, due to variability across subjects and across neurological domains efforts of summarizing this process with precision have been frustrating. Among the most obvious factors that contribute to the extent of spontaneous recovery are infarct size, infarct location, age and pre-stroke disability (Cramer, 2008). Most spontaneous recovery tends to occur within the first three months. While patients with milder deficits achieve spontaneous recovery more quickly than

patients with more sever deficits, the pattern of spontaneous recovery can also differ within the same patient for different functions (Cramer, 2008).

1.1.1 Spontaneous recovery of sensorimotor function in humans

Motor recovery has been among the most often examined because motor impairments belong to the symptoms that are most frequently and precisely diagnosed after stroke (Rathore et al., 2002; Gresham et al., 1995, Langhorne et al., 2009). Motor impairment can be regarded as a loss or limitation of function in muscle control or movement or a limitation in mobility. It is a focus of physiotherapy or occupational therapy in terms of stroke rehabilitation (Langhorne et al., 2009). The natural history of motor recovery is considerably heterogeneous: The first voluntary movements can be seen anywhere from 6 to 33 days after a hemiplegic stroke (Twitchell 1951). The largest improvement occurs in the first 30 days after stroke, though significant progress is still found in patients with more severe deficits up to 90 days after stroke (Wade et al., 198; Duncan et al., 1992, Duncan et al., 1994, Duncan et al., 2005). Studies on arm disability revealed that a maximum of function is reached by 80% of the patients within 3 weeks and by 95% of patients within 9 weeks (Nakajama et al., 1994). Still significant long-term improvement is found if arm function starts to ameliorate 16 weeks after stroke onset (Broeks et al., 1999).

Insights into the underlying remodeling and re-organization processes for functional recovery in the brain after stroke can be obtained in human patients via functional neuroimaging methods and brain mapping. These data suggest that recovery of motor function after stroke leads to brain-wide modifications in neuronal activity patterns and connectivity (Rehme and Grefke, 2012). While initially tissue function and neurophysiological responses are diminished within the injured primary neocortex, cortical function increases over time (Marshall et al, 2000; Calautti et al. 2001, Feydy et al., 2002; Grefkes and Fink, 2011). In terms of good functional outcome one of the major correlates is the degree of recovery of neurophysiological activity in the affected primary cortical areas (Cramer 2008). In other terms: the best behavioral outcomes are associated with the greatest restoration/remodeling of brain function towards the normal state of organization (Ward et al., 2003; Zemke et al., 2003; Ward 2004; Murphy and Corbett 2009). This is true even if the post-stroke behaviour is far from being identical to the pre-stroke motor kinematics. In particular the extent of corticospinal tract integrity is positively correlated to functional recovery as revealed by transcranial stimulation of the motor cortex and its efferents after stroke (Talelli et al., 2006). In general, if an ischemic event occurs, those areas are recruited for structural and functional modification which are either close or functionally related and connected or both. Therefore, after a small stroke, peri-infarct tissue is mainly involved that has similar function. By contrast, after a large stroke, tissue that has similar functions might be only found at more distant sites or in unaffected regions of the contralateral hemisphere, where still enough capacity for structural remodeling remains (Murphy and Corbett, 2009).

1.1.2 The role of the premotor and contralesional motor cortex

Which areas are activated and what they contribute in terms of beneficial re-organization for functional recovery is still under debate: A meta-analysis revealed that activation of premotor areas and the contralesional primary motor cortex are consistent findings (Rehme et al., 2012, Rehme et al., 2013). Interactions between premotor areas and the lesioned primary motor cortex (M1) are directly related to recovery and functional outcome. For example, Johansen-Berg showed that disruption of dorsal premotor cortex activity by transcranial magnetic stimulation (TMS) over both the ipsi- and contralateral hemisphere lead to a deterioration of performance in stroke patients, but not in healthy controls (Johansen-Berg et al., 2002). The exact role of

the activation of contralesional M1 is a subject to controversy: Longitudinal functional MRI studies revealed enhanced neuronal activity in motor-related areas in both hemispheres after a large stroke. But then during the first 12 months post-stroke this activity returns to unilateral levels similar to those of healthy controls for those patients with good motor recovery (Ward et al., 2003). Remaining increased activity in the contralesional M1 was often associated with poor outcome. Further studies have demonstrated that inhibition of contralesional M1 activity using repetitive TMS may lead to ameliorated motor performance of the stroke-affected hand in the subacute and chronic phase (Nowak et al., 2008; Takeuchi et al., 2012). In contrast, Rehme et al., 2010 found that increases in contralesional M1 activity over the first 10 days after stroke correlate with the amount of spontaneous motor improvement in initially more impaired patients. These data suggest a supportive role for functional recovery in the early phase after stroke for the contralesional motor cortex. In addition, disrupting contralesional M1 activity with TMS resulted in a deterioration of motor-performance of the stroke-affected hand of stroke patients with capsula interna infarcts (Lotze et al., 2006). A clear time-, size or lesion-location- dependent influence of the contralesional M1, be it either beneficial or harmful for functional recovery, remains to be demonstrated.

1.1.3 Changes in cortical excitability, lateralized activation and somatotopic re-mapping

For the above described remodeling and recruitment of areas three main forms of reorganization have been described: (1) Increased cortical excitability in cortical regions distant from, but connected to the stroke core; (2) reduced lateralized activation and (3) somatotopic modifications within intact cortical regions.

Increased activity, as a first form of reaction to stroke in areas which before stroke formed a distributed network, has been described many times (Brion et al., 1989; Chollet et al. 1991). This phenomenon occurs in several cortical areas which include motor, language, attention and visual functions (Cramer 2008). Widespread areas of cortical hyperactivity appear days after stroke and diminish within months post incident (Ward 2004). This form of modification in cortical excitability is thought to be a result of the down-regulation of the $\alpha 1$ γ-amino butyric acid receptor subunit and a decrease in γ-amino butyric acidergic inhibition (Neumann-Haefelin et al. 1998).

The second form of reaction to stroke – reduced lateralized activation – reflects the increased activity in the contralesional hemisphere, which reduces the extent of interhemispheric balance as demonstrated in many stroke studies (Weiller et al., 1993, Seitz et al., 1998). Reduced lateralized activation is a common brain response not only seen in stroke but also in other neurological contexts such as epilepsy, traumatic brain injury and multiple sclerosis (Cramer, 2008). The exact function of this reduced laterality remains to be elucidated: It may be just a subtype of the described increased activity as described in (1) or a passive event reflecting a reduced interhemispheric inhibition resulting from the stroke. Another interpretation is that the contralesional hemisphere has to take over functions that were previously based in the ipsilesional hemisphere. Both phenomena, increased cortical excitability and reduced laterality, are related to spontaneous functional recovery (Cramer, 2008). Both are time dependent, increasing in the early weeks after stroke and decreasing over months thereafter. This decrease is greater among stroke patients with stronger functional recovery while the persistent increased activity over both hemispheres is greatest in those patients with the poorest outcome (Cramer et al. 2006; Ward et al., 2003). A relation to increased susceptibility for seizures and phantom pain is possible.

The third response to ischemic injury – somatotopic reorganization – implies that intact cortical regions – in particular within the perinfarct area – reassign their functions which they subserved before stroke and take over function, which have been affected or lost by the ischemic event. Some studies suggest that the largest degree of somatotopic reorganization is associated with very large stroke injuries (Cramer et al., 2006). Such map shifts occur in primary and secondary cortical areas (Byrnes et al., 2001).

1.1.4 Animal models to study stroke induced cortical re-organization on the anatomical and molecular level

As studies in stroke patients have limitations, animal models of stroke have been used to describe remodeling and reorganization processes on the macro and molecular level. Although spontaneous recovery in animals tends to occur earlier (depending on stroke size), imaging and mapping data show a number of analogues between recovery in animals and in humans: Connectivity changes between sensorimotor cortex and deep grey matter structures after middle cerebral artery occlusion (MCAO) in rats were comparable to results in human stroke patients (Van der Zijden et al., 2007). fMRI studies concentrating on the affected upper limb in rats have described a shift in laterality of activation after stroke such that early after stroke, brain activation during affected paw stimulation is mainly in the contralesional cortex, later after stroke activity shifts toward the normal pattern, that is the ipsilesional cortex (Dijkhuzen et al. 2001; Dijkhuzen et al., 2003). Hsu et al., 2006 found that the larger the ischemic insult the stronger the activity in the contralesional motor cortex. In accordance with human studies Van Meer et al., 2012 could show that functional recovery after MCAO in rats was correlated with the extent of preservation or restoration of the ipsilesional corticospinal tract in combination with reinstatement of interhemispheric neuronal signal synchronization and normalization of focal network organization.
New mapping methods allow describing somatotopic map shifts in animals in greater detail: A recent study using light based motor mapping in transgenic mice expressing light-sensitive channelrhodopsin-2 before and after focal ischemic lesions of the forelimb sensorimotor areas revealed decreased motor output in the infarcted area and spatial displacement of sensory and motor maps (Harrison et al., 2013). While strokes in sensory cortex caused the sensory map to move into the motor cortex, a stoke in the motor cortex lead to a compensatory increase in peri-infarct cortical motor output, but did not affect the position or excitability of the sensory maps. In vivo 2-photon calcium or voltage sensitive dye imaging furthermore opens up new possibilities to study the reorganization of complex neuronal networks and their functional relevance for stroke recovery (Winship and Murphy, 2008; Stetter, 2013). Anatomically, different studies have demonstrated that map-shifts and re-mapping can be accompanied by axonal sprouting (Carmichael, 2003), and dendritic spine turnover (Brown et al., 2008, 2009; 2010). Using different tracing techniques, Starkey et al., 2012 could show which neurons take over when functional map shifts occur: If the forelimb motor cortex in rats was destroyed, neurons in the hindlimb area took over to enable functional recovery of the forelimbs. This functional shift was based on sprouting of new axon branches from hindlimb corticospinal fibers into the cervical spinal cord, followed by retraction of the original lumbar projecting axon and thus a conversion of a hindlimb into a forelimb projecting neuron.
Animal studies have also provided first insights on underlying molecular changes. A unilateral infarct is associated with a number of growth related processes, in some cases bilaterally. These events include the induction of inflammatory markers, grow-promoting and inhibiting genes, cell-cycle regulatory genes and genes involved in synaptogenesis, dendritic branching and neuronal sprouting as reviewed elsewhere (Li and Carmichael, 2006; Popa-Wagner et al., 2007).
Three major phases of stroke reaction and repair are often distinguished (Cramer 2006): The

first epoch is the acute reaction to the injury and takes place in the initial hours when modifications become apparent in blood flow, edema, metabolism and inflammation. A second epoch is related to repair, starts in the first days post stroke and is on-going for several weeks. During this epoch spontaneous recovery is seen and endogenous repair related events reach their peak levels. The third epoch begins weeks to months after stroke when spontaneous recovery has reached a plateau and represents a stable but still modifiable chronic phase.

On the molecular level stroke induces neuronal growth-promoting genes in sequential waves post insult to initiate axonal sprouting in the peri-infarct cortex, as initially shown in a rat somato-sensory cortex (barrel field) infarct model (Carmichael et al., 2005): In the early phase immediate early genes and growth related mRNAs such as SPRR1 are induced 3 to 7 days after stroke. Typical growth cone constituents such as GAP43, CAP23 and MARCKS as well as the transcription factor c-Jun are expressed from day 3 onward. Subsequently, the cell adhesion molecule L1, cyclin-dependent kinase inhibitor p21 and embryonic tubulin isoform alpha1 tubulin are induced, followed by the expression of cytoskeletal reorganization genes such as SCG10 and SCLIP. This pattern of growth gene expression described is unique for axonal sprouting as a stroke response compared to expression profiles in neuronal development, peripheral or other CNS injuries (Li et al., 2010). Furthermore, in an early response to stroke (Mattson, 2008; Carmichael, 2012), several neurotrophic factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin 3 (NT-3) as well as fibroblast growth factor (FGF)-2 and insulin-like growth factor (IGF-1), epidermal growth factor (EGF) and glial cell line-derived neurotrophic factor (GDNF) are up-regulated. Each neurotrophic factor species shows a different temporal and cellular distribution pattern(Abe et al., 2000): While GDNF is mainly expressed by neurons, CNTF induction was predominantly observed in astroglia of the marginal region and VEDF gene expression was found in both non-neuronal and neuronal cell types after stroke.
Axonal sprouting not only requires the induction of growth-promoting programs within perinfarct neurons, but also a reduction in the growth inhibitory environment (Carmichael, 2006): Axonal growth inhibition in the adult CNS is mediated through three general classes of proteins: myelin associated proteins (NogoA, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein), extracellular matrix proteins (e.g. chondroitin sulfate proteoglycans) and repulsive cues for growth cones known mainly from development (e.g. ephrins, semaphorins). Interestingly, messenger RNAs for the chondroitine sulfate proteoglycans aggrecan, phosphacan and versican were found to be induced later after stroke than the early and middle phase of the growth-promoting gene expression. A small number of growth inhibitory proteins including Nogo A (Jiang et al., 2009), ephrin A5, semaphoring IIIa and neuropilin 1are induced in the early phase, however, but down-regulation of Nogo receptor components were also seen (Li et al., 2010).
Not only a temporal expression pattern of growth promoting and inhibiting genes can be detected, but also the spatial distribution plays a role to induce the brain´s self-repair processes at the right location: Axonal sprouting e.g. in the peri-infarct cortex takes place in a distinct environment close to but larger than the glial scar. Thus, within the glial scar representing the wall that separates the stroke core from the surviving per-infarct tissue both, growth-promoting and growth inhibiting factors are induced while the growth-permissive and peri-infact cortex shows a reduction of the levels of growth inhibiting molecules such as chondroitin sulfate proteoglycans. In contrast, neurotrophins such as BDNF are highly up-regulated in the growth-permissive penumbra und repressed in the stroke core (Lanfranconi, 2011).

Taken together, the data on the time and space dependent processes of intrinsic repair mechanisms after stroke suggest a critical period or time window, in which the CNS recruits factors for plasticity that enhance functional recovery. One of the most crucial questions that has to

be addressed from a clinical perspective is whether this period characterized by map shifts, fiber growth and major functional and structural changes is also the time window in which rehabilitative interventions should be initiated. We now give an overview on rehabilitative and repair strategies with an emphasis on timing, kind and intensity.

1.2 Strategies to enhance plasticity after stroke

1.2.1 Growth and plasticity enhancing treatments

Since the discovery of nerve growth factors and factors that prevent neuronal outgrowth and survival, it became a goal in experimental animal studies to apply or induce growth-promoting factors and inhibit the inhibiting ones. Several preclinical studies have examined various growth factors, hormones and cytokines with the aim to enhance motor rehabilitation – including prominent candidates such as nerve growth factor, glia (GDNF) and brain derived neurotrophic factor (BDNF), insulin-like growth factor, erythropoietin and the granulocyte colony-stimulating factor. All have met with variable levels of success in animal models; some initial clinical studies have started (The BDNF study group, 1999; Nagahara and Tuszynski, 2011).
In adult rats with large strokes, the administration of BDNF resulted in improved recovery rates (Schabitz et al., 2004), while the beneficial effect of rehabilitation on the improvement of forelimb function was prevented in animals treated with a BDNF antisense oligonucleotide (Ploughman et al., 2009). The translation of these results into clinical trials remains challenging and is a matter of safety concerns: In the case of BDNF applied as a neuro-protective agent after stroke, the administration of very large quantities would be necessary as well as repeated dosing to overcome the limited amount of protein that reaches the CNS, even with transient disruption of the blood-brain barrier after stroke. The adverse effects of these high dosages have not been extensively studied in animal models (Nagahara and Tuszynski, 2011). Furthermore, the largest clinical trial of erythropoietin therapy revealed that, compared with placebo, erythropoietin administration was associated with an increased risk of mortality in patients with acute stroke (Ehrenreich et al., 2009).
Other experimental approaches to enhance the intrinsic regeneration ability of CNS axons include injecting cAMP analogs to influence intracellular signaling pathways (Hannila and Filbin 2008), knock down of the protein synthesis inhibitor PTEN (Liu et al., 2010) or blocking the small GTPase RhoA (Ellezan et al., 2002).
Promising results have also been gained if inhibition of neuronal plasticity and outgrowth was decreased either by (1) digesting growth restricting ECM proteoglycans with enzymes such as chondroitinase ABC or (2) by blocking the growth inhibitory protein Nogo A (3) or by grafting growth permissive cells. The bacterial enzyme chondroitinase ABC digests the glycosaminoglycan chains of the chondroitin sulfate proteoglycans (CSPGs) which are part of the extracellular matrix and usually up-regulated in astrocyctes and oligodendrocytes after CNS injury (Garcia-Alias and Fawcett, 2012). Chondroitinase ABC treatment reduces scar formation and enhances axonal regeneration and sprouting as first shown in several studies after experimental spinal cord injury (Moon et al., 2001; Bradbury et al., 2002; Huang et al., 2006). After stroke, chondroitinase ABC administration promoted functional recovery (Hill et al., 2012; Starkey et al., 2012). Furthermore, Soleman et al., 2012 could demonstrate that delayed chondroitinase ABC microinjections into the cervical spinal cord induce localized plasticity of the forelimb sensorimotor spinal circuitry without effects on the cortical peri-infarct region.

Inhibition of Nogo-A Signaling in animal models of stroke

The well-studied protein Nogo-A, a transmembrane protein of about 1200 amino acids including a C-terminal 200 amino acid reticulon (RTN) domain, is involved in several cellular and molecular events contributing to the failure of CNS axons to sprout and reconnect after CNS injury. Function-blocking antibodies against Nogo-A, Nogo receptor (NgR1)-blocking peptides, antibodies against the Nogo receptor subunit Lingo-1, or pharmacological blockade of the signal transducer RhoA and ROCK have been administered in various laboratories in different stroke and spinal cord injury models in rodents and primates (Pernet and Schwab, 2012 for review). Enhancement of behavioral recovery in a variety of sensory-motor tasks as well as anatomical evidence of fiber growth, increased plasticity and re-organization within the cortex, brain stem and spinal cord have been reported (Zörner and Schwab 2010 for review). Despite different approaches to interrupt Nogo-A signaling, a high degree of similarity in terms of functional recovery and hardware changes in the CNS was found among research groups and injury models. Acute intrathecal anti-Nogo A antibody infusion over 2 weeks after stroke, with an application starting early after incident (Wiessner et al., 2003; Tsai et al., 2007), or delayed application starting 9 weeks after stroke in adult rats (Tsai et al., 2011) significantly improved forelimb function and was correlated with a significant increase of midline crossing corticospinal fibers originating in the unlesioned sensorimotor cortex. Robust sprouting of new projections from contralesional brain regions into subcortical structures as well as functional reorganization of contralateral sensorimotor areas were reported after anti-Nogo-A immunotherapy in rats (Markus et al., 2005; Cheatwood et al., 2008). Those newly sprouting cortico-efferent axons terminated in the red nucleus, pontine nuclei and spinal cord. A similar effect was found by down-regulation of the Nogo receptor NgR using adenovirus-mediated RNA interference (Wang et al., 2010) or NgR or Nogo-A/B knockout mice (Lee et al. 2004). Anti-Nogo-A immunotherapy was also associated with increases in dendritic length, complexity, and spine density, both in the lesioned and contralesional hemisphere (Papadopoulos et al., 2006). Functional MR-imaging 8 weeks after unilateral middle cerebral artery occlusion revealed adaptations in the somatosensory system of rats in the anti-Nogo-A antibody treatment group (Markus et al., 2005). Nevertheless anti-Nogo-A immunotherapy is not neuroprotective in the sense that it would reduce stroke lesion size as reported for anti-MAG immunotherapy (Irving et al., 2005). This opens the therapeutic window for anti-Nogo-A immunotherapy in the subacute and even chronic phase.

The described in vivo experiments represent essential preclinical tests to validate the efficiency and safety of intrathecal Nogo-A antibody administration. Three different anti-Nogo-A antibodies (IN-1, 11C7, 7B12) have proved efficient in enhancing axonal regeneration and outgrowth both in vitro and in vivo. In collaboration with Novartis Pharma, a human anti-human Nogo-A antibody has been developed and tested in extensive toxicological studies with intrathecal antibody application in rodents and primates.

In a Phase I clinical trial (http://clinicaltrials.gov/ct2/show/NCT00406016) with 52 acutely injured para- and tetraplegic patients in Europe (European Multicenter Study about Spinal Cord Injury, EMSCI, www.emsci.org) and Canada pharmacokinetics, safety, tolerance and dosing of intrathecal delivery of the antibody were investigated. The tolerance has been excellent without any adverse effects ascribed to the anti-Nogo-A antibody (Abel et al., 2011). A placebo-controlled Phase II clinical trial is currently in preparation. Anti-Nogo antibodies are also in clinical trials or in preparation for clinical trials for other neurological indications such as multiple sclerosis and amytrophic lateral sclerosis (ALS). For ALS GlaxoSmithKline (GSK) has also developed a humanized anti-Nogo-A antibody (GSK1223249). In a Phase I clinical trial, the intravenous injections of GSK1223249 were well tolerated by the 76 patients enrolled in the study (Pradat et al., 2011).

Several additional molecules restricting axonal growth in vitro have been identified including

ephrins, netrins, semaphorins and OMgp (oligodendrocyte myelin glycoprotein)(Schwab et al., 1990, Schwab et al., 1993; Schwab, 2010). Their role in vivo after stroke has to be evaluated. How much growth and plasticity of the adult, stroke-injured CNS can be enhanced by single or combined manipulations of growth promoting or inhibitory mechanisms, and if there is a danger of chaotic growth and formation of wrong connections is currently unknown.

Finally, grafting growth permissive cells, such as bone-marrow mesenchymal cells, cord blood cells, fetal cells and embryonic cells as a form of restorative therapy have been studied in animals (Chopp and Li, 2002). E.g. cultivated bone-marrow stromal cells from donor rats were stereotaxtically implanted into the peri-infarct area in rats resulting in significant recovery of somatosensory behaviour. In a first small study, 5/30 stroke patients who received autologous bone-marrow mesenchymal cell transplantation showed beneficial effects in clinical stroke scores (Bang et al., 2005). Such cell-based therapies could influence endogenous neurogenesis, axonal sprouting and synaptogenesis in ischemic brain tissue (Zhang and Chopp. 2009), although their effects may be primarily immune-modulatory or neurotrophic. More detailed and systematic studies are certainly needed.

1.2.2 Rehabilitative training in clinical and experimental studies

The brain, including the motor system, learns by repetition and training. Many basic mechanisms, however, are still poorly understood, and rehabilitative training is largely evidence-based medicine (European Stroke Organization Guidelines 2008). Nevertheless there are no generally accepted guidelines and no definite recommendations concerning the timing, kind and intensity of rehabilitative training. Clear end point data and randomized controlled clinical trials are often lacking. Furthermore, stroke recovery is a complex process that probably occurs through a combination of restoration, substitution and compensation of functions. For this reason it has been also difficult to translate results from rehabilitative studies in animals to recommendations for rehabilitative schedules in human stroke patients. A majority of clinical studies has been conducted in chronic stroke patients (>6 months after the stroke) as recruitment of these patients was easier and baseline performance had stabilized (Krakauer et al., 2012). These circumstances lead to functional outcome measurements probably gained largely from compensatory techniques to improve skills for daily living. In contrast, animal studies had a stronger focus on enhancing impairment with more or less detailed analysis how much of the functional recovery was restoration of baseline (motor) function or compensation. Furthermore, the time courses of motor recovery differ among animal and human studies: While recovery in rodent models reaches its maximum around 4 weeks after stroke, human stroke survivors complete most of their recovery within 3 months (Dimyan and Cohen, 2011; Krakauer et al., 2012).

Early versus delayed training

A consensus exists that the effects of early training, - whereby 'early' should be starting at 1-2 weeks in animals, not earlier (see below), - exceed effects of delayed training in terms of functional recovery in both, animals and humans (Nudo, 2006; Murphy and Corbett, 2009; Langhorne et al., 2011; Krakauer et al., 2012). In animal studies, behavioral training after ischemic injury is most effective for restoring behavioral performance, peri-infarct neurophysiological maps and enhanced neuroanatomical changes in the ipsi- and contralesional hemisphere when introduced within the first week of injury (Nudo 2006). In a rat middle cerebral artery occlusion (MCAO) stroke model it was demonstrated that functional outcome and dendritic branching patterns in the contralesional hemisphere were restricted when rehabilitative training was initiated 14 and 30 days post insult (Biernaskie et al., 2004). In another study by Hsu and Jones 2005, rats were trained in a skilled forelimb reaching tasking starting 4 or 25 days

post stroke. Reaching performance was significantly enhanced in the early trained group. In a small ischemic insult in M1 in squirrel monkeys delayed training resulted in a large decrease in spared hand representation during the spontaneous recovery period that persisted following the delayed training (Barbay et al., 2006).
Concerns about initiating therapy too early following stroke arose from studies where lesion size and cell death rate were seen to be exaggerated after early excessive use of the impaired forelimb in rats while the unimpaired forelimb was casted (Kozlowski et al., 1996). One cause for increased lesion size following early excessive limb training might be NMDA-mediated excitotoxicity in the already hyperexcitable peri-infarct region (Humm et al., 1999). In closer resemblance to clinical practice were animal studies, where training or enriched rehabilitation was initiated a few days after stroke. In these cases early intervention (1-3 days post stroke) again was associated with increased cell-death but also with much improved motor performance on the long-term (Risedal et al., 1999; Farrell et al. 2001). Here, neuronal cell death may be part of a pruning effect in which non- or dysfunctional neurons are eliminated early due to a use-dependent selection. In summary, the overall consensus from animal data is that initiating rehabilitative training 5 or more days after stroke is mostly beneficial and has no adverse effects (Krakauer et al., 2012).

CIMT, robot assisted training and electrical devices to stimulate the rehabilitation process

For human stroke patients two advanced rehabilitative approaches have proven beneficial for functional outcome: constraint-induced movement therapy (CIMT) and robot-assisted training for upper limb function (Langhorne et al., 2009; Mehrholz et al., 2012; Liao et al., 2012). Extensive preclinical studies in rodents and primates have preceded both rehabilitative strategies (Taub et al., 2002). When somatic sensation is surgically abolished from a single forelimb in a monkey, the animal avoids the usage of this forelimb in the free situation, but monkeys can be induced to use the de-afferented extremity by restricting movement of the intact limb continuously for a period of days. This concept was successfully brought into the clinics when chronic stroke patients wore a sling or cast on their less affected arm during 90% of their waking hours for 14 days (Taub et al., 1993). These patients showed a significant increase in the skill and quality of movement as measured by two laboratory tests and a much larger increase in real-world arm use over the period of these two weeks than the unrestricted control group. Two studies addressed the question of intensity and timing for CIMT: In the VECTORS study (Dromerick et al., 2009), 52 stroke patients were randomized at about 10 days post stroke to two levels of intensity of CIMT or standard upper extremity therapy. Intense meant 3 hours of CIMT versus 2 hours of shaping therapy. After 90 days the motor outcome was worse for the more intensive CIMT group, although there had been no difference at 30 days. This result reflects the fact that too intensive CIMT can turn into an adverse situation for both the patient and the therapist. In the much larger EXCITE study (Wolf et al., 2006) patients started CIMT therapy 3 to 9 months post stroke and showed greater motor recovery than the usual care group. In addition Lang et al., 2013 revealed that improvements in existing motor abilities were possible with both early (3 to 9 months post stroke) and delayed (15 to 21 months post stroke) application of CIMT. However, significant reacquisition of the ability to complete tasks was only detected with early CIMT treatment.

A number of arm and also hand training robots have been developed recently with the aim to allow very intense training without continuous, costly physiotherapy assistance. In the most modern set-ups, training devices are combined with interactive video games that can boost the motivation of the patient for the training and facility e.g. precision movements (e.g. grasping eggs and putting them into a basket). The number of well controlled and standardized outcome

studies is still very limited. However, differences are discriminated between recovery of specific movements under 'laboratory conditions' and functional gains for daily life activities (Mehrholz et al., 2012). Such studies are needed to exactly know the specific advantages (and potential drawbacks) of robot assisted rehabilitation in stroke (Aisen et al., 1997; Balasubramanian et al., 2010; Mehrholz et al., 2012).

Therapeutic approaches which directly stimulate the PNS or CNS electrically or by magnetic pulses may enhance neuroplasticity during poststroke rehabilitation (Dimyan and Cohen, 2011). Numerous research groups have examined the stimulation of the CNS, specifically the primary motor cortex (M1), by noninvasive approaches such as transcranial magnetic stimulation (TMS) and direct current stimulation as well as experimentally in animals by the implantation of electrodes. Several studies showed that an increase of the excitability in the stroke-affected ipsilesional M1 by electrical devices resulted in improved motor outcome (Hummel et al., 2005; Malcolm et al., 2007, Ameli et al., 2009; Koganemaru et al., 2010). The mechanisms of action of these techniques are under investigation but might involve changes in synaptic activity, gene expression and increases in neurotransmitter, receptor and neurotrophin levels (Dimyan and Cohen, 2011) or even enhanced fiber sprouting (Martin 2012). Understanding these mechanisms may provide the basis for novel approaches using closed-loop brain machine interfaces (BMIs) that define optimal stimulation parameters from a priori developed experimental models and correctly modulate ionic currents and extracellular electric fields to provoke and guide plastic changes of the CNS (Gonzalez Andino et al., 2011).

1.3 Combination of different repair and rehabilitation strategies

To maximize the effectiveness of rehabilitative therapies after stroke, it is critical to define when the brain is most responsive to sensorimotor input or extrinsic application of plasticity promoting reagents. This becomes particularly important if different rehabilitative approaches are combined.

In one of the first proof of concept studies for a critical period of heightened neuroplasticity, stroke rats were exposed to an enriched environment in combination with daily sessions of grasping training. The most significant gains in the recovery of forelimb reaching ability were achieved when rehabilitation was initiated early, i.e.5 days after stroke as compared to 14 and 30 days after stroke. Recovery was associated with increased dendritic branching of layer V motor cortex neurons in the unlesioned hemisphere – a response that was not detected when rehabilitation was delayed by 30 days (Biernaskie et al., 2004).

A few recent studies in which regenerative therapies and rehabilitation have been combined have been conducted since then. These experiments suggest that designing the combination and their temporal pattern of administration are not going to be trivial (Garcia-Alias and Fawcett, 2012; Starkey and Schwab, 2012).The different experiments have revealed a beneficial combinatorial effect, a detrimental effect, no effect at all, or an effect that depends on the relative timing of plasticity treatment and rehabilitation. Beneficial effects were described in spinal cord injury rat models when agents against inhibitory molecules in the CNS were combined with growth promoting reagents: Garcia-Alias et al., 2011 reported that the combination of Chondroitinase ABC with neurotrophin NT-3 and an increased expression of the NR2D subunit of the NMDA receptor resulted in better body stability and interlimb coordination compared with the single treatment groups. The behavioral data were correlated with the highest number of sprouting axons in the spinal cord and multisynaptic responses in the motor-neurons. Similar results could

be found if anti-Nogo-A antibodies were combined with NT-3 and the NMDA-NR2D subunit (Schnell et al., 2011). Furthermore, the combinatorial treatment of acutely applied anti-Nogo-A antibody followed by delayed Chondroitinase ABC treatment starting 3 weeks after spinal cord injury, and forelimb grasp training starting at 4 weeks was much more effective in terms of functional recovery, sprouting and axonal regeneration than the single treatments (Zhao et al., 2013). In rats with large cortical strokes, inosine, a substance which was shown to improve fine motor control after stroke (Zai et al., 2009), augmented the effects of the Nogo receptor blocker NEP1-40 in the restoration of skilled reaching abilities in rats. Similar functional improvements were seen when inosine was combined with environmental enrichment (Zai et al., 2011).

Several recent experiments – mainly in spinal cord injury - have combined growth-promoting agents with rehabilitative training with somewhat different results: Garcia-Alias et al., 2009 investigated whether chondroitinase-induced plasticity combined with physical rehabilitation promotes recovery of manual dexterity in rats with cervical spinal cord injury. While CSPG digestion combined with forelimb-specific rehabilitation lead to improved manual dexterity, animals treated with chondroitinase ABC in combination with environmental enrichment improved in ladder walking but performed much worse in skilled forelimb tasks than untreated control animals. In a second investigation by Maier et al., 2009 adult rats with large but incomplete cervical spinal cord injury received anti-Nogo A antibodies and simultaneous daily forced treadmill training. The simultaneous rehabilitative therapy clearly worsened the functional outcome compared with either treatment alone. When the forced treadmill training was delayed, however, for two weeks after the end of the antibody treatment a very good functional outcome was obtained (Marsh et al., 2010). In contrast to these results in spinal cord injured rats, combination of Nogo receptor blockade with skilled forelimb training in stroke lead to a greater degree of recovery than when either of the treatments were applied alone (Fang et al., 2010).

No additive or adverse effects were reported by Boyce et al., 2007 when neurotrophins were combined with rehabilitative training in spinal cord injured cats. Administration of pharmacological neuromodulators such as amphetamine and cholinergic agonists in combination with rehabilitative training are a matter of debate: Early animal research had suggested a beneficial effect of amphetamine in recovery of motor function after stroke which could not be sufficiently reproduced in recent human and animal studies (Krakauer et al., 2012). Only for the antidepressant fluoxetine, a serotonin-selective reuptake inhibitor, which was applied from 9 days post stroke to 3 months in a human stroke study, an impressive degree of increased motor recovery was found when combined with rehabilitative training (Chollet et al., 2011). For all these studies and their quite diverse outcomes, better knowledge of the neurobiological phenomena and mechanisms triggered by the injury, the spontaneous reaction of the nervous tissue to it, and by the different pharmacological and behavioral interventions is urgently required.

1.4 Future directions for designing optimal rehabilitation schedules

How can we better understand the neurobiology of rehabilitation? What can we learn from the above mentioned animal and clinical studies to improve current rehabilitation schedules for the best possible recovery after stroke? The presence of critical time windows for the application of growth and plasticity promoting agents and of training-dependent plasticity suggests that careful consideration of rehabilitation onset times, tailored training to the type and extent of stroke and the patient´s history are required. Potential future rehabilitation schedules after stroke may therefore include the following '3 step model' (Figure 1):

(1) Determination of the metabolic and plastic status of the brain by using state-of-the-art imaging technologies and metabolic markers
(2) Enhancement of the plastic status of the brain by the application of growth and plasticity-promoting factors
(3) Selection and stabilization of newly formed functional connections by rehabilitative training

One obstacle of the implementation of the optimal restorative therapies is the heterogeneity of stroke as injury location and size differ widely from one patient to another. The ability to assign the right therapy to the right patient would maximize treatment effects. Although clinical scores and a number of imaging methods exist for evaluating the state of the central nervous system and its function after stroke as reviewed elsewhere (Burke and Cramer, 2013), these approaches are often insensitive, cost intensive and have logistical difficulties. Nevertheless, neuroimaging is not only essential for the establishment of acute stroke diagnosis but can also serve as a powerful tool for the characterization of disease progression and monitoring of the response to rehabilitative interventions. Diffusion-weighted imaging (DWI) and perfusion-weighted MRI(PWI) are widely available MRI modalities that provide valuable information about the tissue characteristics of the ischemic core but also of the tissue at risk in the penumbra (Merino and Warach et al., 2010; Fisher and Bastan et al., 2012). Further work is needed to optimize the characterization of penumbra imaging for patient triage into adjusted treatment groups. In the near future we expect to learn if penumbra imaging or other early imaging features provide predictive value of critical time windows in which therapeutic interventions should be initiated or maintained and allow stratification of patients into groups for specific types of therapies.
Biomarker profiles in blood and CSF samples could bring a tremendous advance and are currently a focus of genomic and proteomic profiling studies and of systems biology in several laboratories (Stuart et al., 2010; Hemphill et al., 2011, Whitley et al., 2012). In this regard, a biomarker or a specific combination and profile of biomarkers may not only speed up diagnosis and initiation of acute stroke treatment but may also help to classify and categorize patient groups for prediction of outcome and target the right rehabilitative approach to those stroke patients who would benefit the most.

Why do we suggest a temporal sequence of first enhancing the plastic state by growth promoting agents followed by a phase of rehabilitative training in our '3 Step Model'?
The current data suggest that the CNS reacts to the injury by an activation of growth and plasticity mechanisms which, however, seem to also represent a vulnerable phase in which forced activity can be harmful: This phase includes a period of GABA-mediated tonic inhibition, which may also be necessary in the first days after the stroke to limit an expansion of the infarct size (Clarkson et al., 2010), as well as homeostatic plasticity mechanisms, which ensure that neurons receive an balanced amount of synaptic input (Murphy and Corbett, 2009). Intrinsic growth and plasticity as well as exogenous enhancement of growth will lead to the formation of a large number of new connections within and between different areas of the injured CNS. In analogy to the situation in early postnatal development, many of these connections may be weak and imprecise. The functionally meaningful ones will now have to be selected and stabilized, while the malfunctional ones should be pruned, in the next, activity-dependent phase of the recovery process.

In the last step of recovery that is based mainly on rehabilitative training the spared and the new circuitry of the CNS is shaped by selection and stabilization of functional connections and pruning of the non-functional ones. Hebbian learning rules might play a crucial role in this

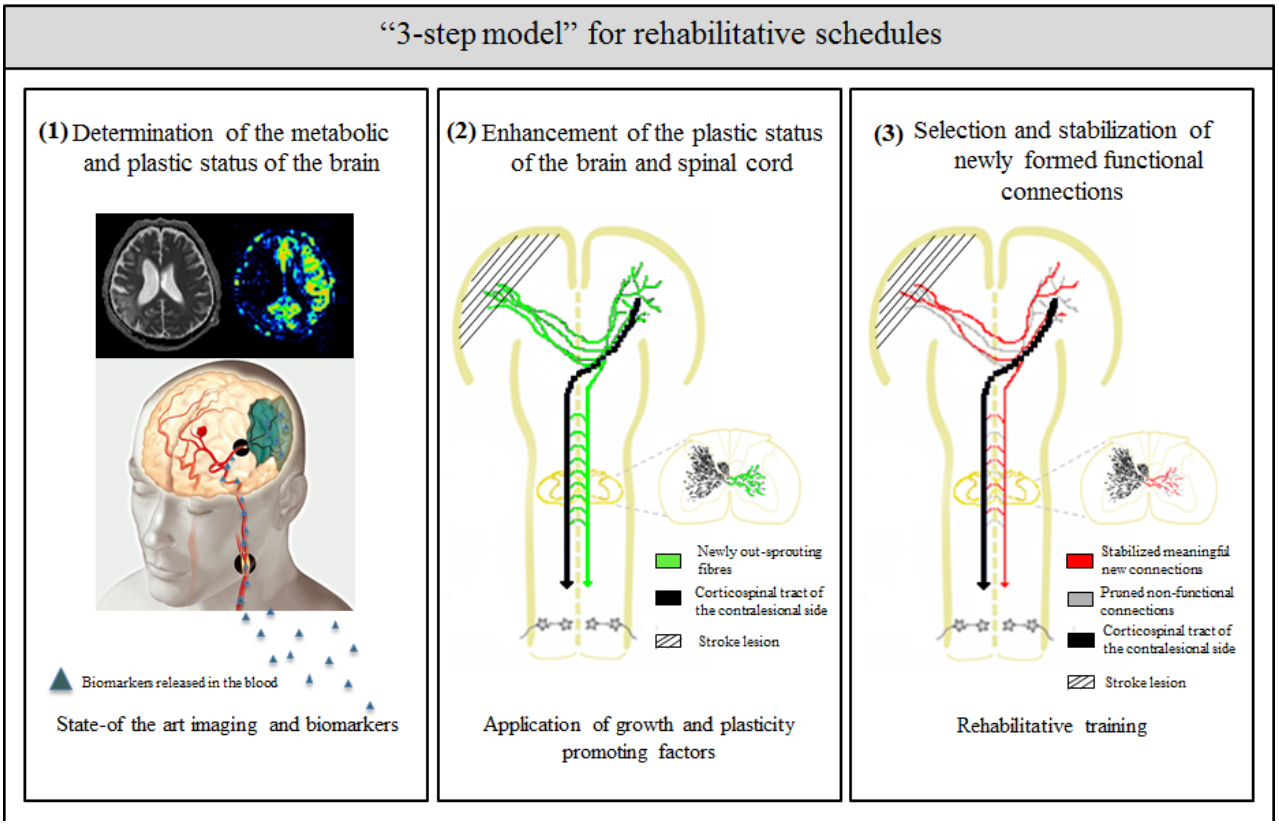

Fig. 1: Schematic overview of the '3 step model' - as a possible roadmap for designing future rehabilitation schedules: (1) Determination of the metabolic and plastic status of the brain by using state- of the art imaging technologies (image taken by the Akashi Municipal Hospital, Japan) and biomarker profiles in the blood and CSF. (2) Enhancement of intrinsic repair and plasticity mechanisms in the ispi- and contralesional hemisphere as well as the spinal cord by application of growth and plasticity-promoting factors such as anti-Nogo-A antibody or Chondroitinase ABC. (3) Selection and stabilization of newly formed functional connections and pruning of non-functional ones by rehabilitative training.

step in the sense that Hebbian plasticity mechanisms redistribute synaptic strength to favor the wiring of pathways that are coincidently active (Murphy and Corbett, 2009). Motor learning in development is a very protracted process, requiring huge numbers of repetitions over a period of many weeks and months. Much too less is known today on the optimal time and intensity requirements for rehabilitation learning. To distinguish optimal rehabilitation schedules from less beneficial ones, strict criteria for functional outcome have to be defined that discriminate compensation and substitution from real restoration of previously impaired function. Much remains to be learned and applied in this fascinating and medically most important field of stroke rehabilitation at the interface between basic neuroscience and clinical neurology.

1.5 References and Notes

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Chapter 2

State-of the art techniques to study reorganization and to manipulate regained motor function after stroke

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Abstract

Current stroke research faces the same challenge as neuroscience: To transform correlative findings in causative ones. Research of recent years has shown the tremendous potential of the central nervous system to react to noxious stimuli such as a stroke: Increased plastic changes leading to re-organization in form of neuronal rewiring, neurogenesis and synaptogenesis, accompanied by transcriptional and translational turn-over in the affected cells, have been described both clinically and in experimental stroke research. However, less attempts have been made to connect distinct plastic remodeling processes as causative features for specific behavioral phenotypes. In this chapter we review current state-of the art techniques for the examination of cortical reorganization and for the manipulation of neuronal circuits as well as techniques which combine anatomical changes with molecular profiling. These tools may be useful to close the loop from our understanding of stroke pathology to behavioral outcome and may allow to discover new targets for therapeutical approaches.

Although huge efforts have been made in recent years, both by clinicians and by basic researcher, we still have limited insights into a disease such as stroke (the second most common cause of death after heart diseases worldwide) preventing us from developing specific cures and resulting in poor statistical numbers: Of 15 million people suffering from a stroke every year, a third dies, a third remains permanently disabled and a third recovers as the stroke itself has not been too devastating. On the clinical side stroke units have been created, which combine experts in intensive care medicine, neurology, physiotherapy and speech therapy, to accelerate and coordinate the diagnostic and therapeutical processes aiming at improving recovery rates for the patients. According to the neurologists' saying 'time is brain' even mobile units have been established to bring the hospital to the patient (Walter et al., 2012) in order to increase the number of patients being eligible for the only currently approved treatment option, the application of the enzyme tissue plasminogen activator (tPA) within a time window of 4.5 hours after stroke onset (Hacke et al., 2008).
On the side of basic research, studying the neurobiology of stroke, we seem to be stuck in a 'black box' situation: we have accumulated data showing the brain's tremendous capacities to reorganize by synaptogenesis and even neurogenesis, by neuronal circuit rewiring and new circuit formation. We find cortical map shifts and hyperactive brain regions after stroke, we detected genetic and proteomic turn-over within a distinct spatiotemporal profile and sequence of events. However, we have failed so far, to transform pure correlative data into causative ones, meaning, we have to be able to causatively connect plastic remodeling processes in the brain to distinct behavioral outcomes. This will not only allow us to form a new understanding of the brain's functional status after stroke, but also opens up possibilities to develop and test the efficiency of new therapeutic approaches. Today's basic stroke research is part of neuroscience that faces the challenges to first describe the broad morphological features, then study fine cellular and molecular events, find genes which are active in one neuron or cell-type but not in another and bring it back to the behavioral phenotype. But as the philosopher of science Karl Popper might have argued, before we can provide answers, we need the power to ask new questions. In recent years new technology has been designed which is starting to fill the gap between correlative and causative research.
The aim of this chapter is to introduce state-of the art technology for the examination of cortical reorganization, manipulation of neuronal circuits and techniques which combine cytology with molecular profiles - all tools, which may elucidate not only our understanding of stroke pathology but also help to identify crucial anchor points for new therapeutic interventions.

2.1 State-of the art techniques to study cortical reorganization after experimental stroke

A classical approach to study cortical reorganization and to find first hints if projections in the motor cortex are functionally relevant, is intracortical microstimulation (ICMS) and surface stimulation with electrode arrays. This technique applies electrical stimulation of cortical sites to induce e.g. stimulus-evoked movement responses, which can be detected visually, by EMG responses or by usage of accelerometers. Several studies have used this technique to either examine cortical map shifts after spontaneous recovery or different therapeutical applications (Emerick et al., 2003; Starkey et al., 2012; Lindau et al., 2014), or used the stimulation itself as a method to increase plastic processes (Brus-Ramer et al., 2007). However, ICMS has its disadvantages such as the inability to selectively target neuronal subtypes as well as the indiscriminate activation of axons of passage. Furthermore, due to electrode penetration intracortical electric stimulation remains an invasive procedure causing tissue damage (Ayling et al., 2009). ICMS is limited to perform cortical representation of body function at a distinct time

point after stroke constraining longitudinal experiments within the same animal.
Another technique to study in particular sensory map shifts is millisecond-timescale voltage-sensitive dye (VSD) imaging which unlike functional fMRI and intrinsic optical signal imaging measures electrical activity with relatively high spatial and temporal resolution (Mohajerani et al., 2013). VSD imaging has recently be applied to measure spontaneous activity over large regions of the mouse cortex to reveal fast, complex, localized and bilaterally synchronized patterns of depolarization (Mohajerani et al., 2010). In a study by Ghosh et al., 2010 VSD imaging was used to show the expansion of the forelimb sensory map towards parts of the hindlimb cortex after a large thoracic spinal cord injury, indicating incorporation of axotomized hindlimb neurons into sensory circuits of the forelimb. In another study by Brown et al., 2009 the function of the sensorimotor cortex was visualized with VSD imaging. The mouse forelimb sensory cortex was targeted by stroke leading to a new sensory representation in the territory previously occupied by the forelimb motor cortex. VSD imaging revealed slower kinetics in remapped sensory circuits accompanied by high levels of dendritic spines as visualized with two-photon microscopy.
A new non-invasive strategy to study the reorganization of the motor cortex after stroke in the same animal over time is light-based motor mapping: This technique makes usage of the possibility to stimulate neurons by light, either by uncaging neurotransmitters (Shepherd et al., 2003; Luo et al., 2008) or by directly activating light-sensitive channels, such as channelrhodopsin-2 (ChR-2). Ayling et al., 2009 used transgenic channelrhodopsin-2 mice which express ChR-2 in layer 5B pyramidal neurons of the motor cortex. Thus, light-based stimulation directly targets corticofugal cells, enabling the analysis of their contribution to motor cortex topography. Light-based motor mapping has the advantage of sampling stimulus-evoked movements at hundreds of cortical locations in mere minutes objectively and in a reproducible manner (Harrison et al., 2012). It is faster and less invasive than electrode-based mapping and can be combined with intrinsic signal imaging in animals with cranial window preparations (Harrison et al., 2013). In addition it enables repeated mapping of the motor cortex over a timescale of minutes to months, opening up possibilities to examine the dynamics of movement representations at distinct conditions such as learning over time, pharmacological intervention or reorganization before, during and after cortical damage. In a first study by Harrison et al., 2012, light-based motor mapping revealed a functional subdivison of the forelimb motor cortex based on the direction of movement evoked by brief light pulses (10ms), while prolonged stimulation (100-500ms) resulted in complex movements of the forelimb to specific positions in space. In a follow-up study (Harrison et al., 2013) light-based mapping was for the first time used to perform a longitudinal experiment studying the reorganization of the sensorimotor cortex after a focal sensory stroke. The sensory stroke caused the establishment of a new sensory map in prior parts of the forelimb motor cortex, which preserved its center position but became more dispersed.

However, although all described mapping approaches are powerful tools, they can only provide information about map shifts and representation of general movement dynamics. They stay far beyond cellular resolution and do not allow to study local neuronal circuitry or single neuron contribution to neuronal networks. In particular after stroke it is not clear how activity in single neurons changes in relation to cortical map shifts. The analysis of single neurons in relation to the neuronal circuit in which they are embedded may elucidate whether stroke-induced plasticity is a result of the capacity of surviving neurons to process multiple functional streams. In vivo two-photon calcium imaging is a potent method which allows not only to study activity of a single neuron or ensembles of neurons in a network, it also enables cell type and neuronal sub-type specific analysis. Only a few in vivo two-photon calcium imaging studies focusing on neuronal reorganization and circuit rewiring after stroke have been conducted so far. In an acute in vivo calcium imaging experiment during the induction of a transient global ischemia

model in mice, Murphy et al., 2008 saw a wide spread loss of mouse somatosensory cortex apical dendritic structure during the phase of ischemic depolarization. This was accompanied by increased intracellular calcium levels which coincidently occured with the loss of dendritic structure. In a second study by Winship and Murphy, 2008 in vivo two-photon calcium imaging was used to study how response properties of individual neurons and glia cells in reorganized forelimb and hindlimb functional somatosensory maps modified during the recovery period from ischemic damage in the sensory cortex. However, all studies have been conducted in animals under anesthesia which itself influences neuronal activity. An experiment which examines single neuron activity in the behaving animal before stroke and during the recovery phase after insult is lacking so far.

2.2 State-of the art techniques for the manipulation of neuronal circuits

In a 1979 Scientific American article Nobel laureate Francis Crick stated that the major challenge facing neuroscience was the need to control one type of cell in the brain while leaving others unaltered. And in a lecture from 1999 he further confined: 'One of the next requirements is to be able to turn the firing of one or more types of neuron on and off in the alert animal in a rapid manner. The ideal signal would be light, probably at an infrared wavelength to allow the light to penetrate far enough. This seems rather farfetched but it is conceivable that molecular biologists could engineer a particular cell type to be sensitive to light in this way' (Crick, 1999). Manipulation of neuronal circuits or single neurons has two prerequisites: Manipulation has to be quick and very specific. Over the years a very diverse set of tools has been developed to manipulate the activity of individual cells and subtypes in the alert behaving animal. The first constraint for specific manipulation implies a high spatial control allowing to selectively modulate a particular cell type (e.g. a parvalbumin positive interneuron) or a distinct anatomical region (e.g. layer 5 pyramidal cells in the sensorimotor cortex). To achieve this aim researchers have either created transgenic mouse lines or locally injected viruses with cell-type specific promotors (Rogan and Roth, 2011). These promotors induce gene expression directly - as in the case of transgenic mice - or indirectly via tet-on/off or Cre-flox systems.
The Tet system uses at least two viral vectors plus an antibiotic drug which in a sequential way like a domino effect activate each other to induce the transcription and translation of the gene of interest: a tissue specific promoter initiates the expression of a transcription factor, either the tetracycline transactivator (tTA) or the reverse tetracycline transactivator (rtTA). The tTA or rtTA then becomes the key player for the transciption of a tetracyclin response element (TRE) promotor, which in dependence of the presence of tretracycline or doxycycline drives the expression of the gene of interest. Expression of the gene of interest is fully reversible as administration or removal of tetracycline or doxycycline will turn expression on or off. In a study by Kinoshita et al., 2012 a Tet-on system was used to selectively express the synaptotoxin tetanus toxin in propriospinal (PN) neurons innervated by the motor cortex. The researchers could show that upon doxycycline administration in the drinking water reaching performance of monkeys significantly declined due to temporal blockade of the motor cortex–PN–motor neuron pathway.
The Cre-flox system functions similarly requiring two transgenes: A tissue specific promotor regulates the expression of Cre recombinase, an bacteriophage enzyme which recombines DNA at specific recognition sequences called loxP sites. Cre recombinase then excises DNA whithin two loxP sites ('floxed'). As in most cases floxed-stop constructs are knocked-in by homologous recombination to a gene of interest(Rogan and Roth, 2011), the stop signal is excised in the presence of Cre and the transgene expression can be initiated. As Cre-mediated expression

only occurs in cells expressing Cre, which are also those in which the tissue-specific promotor is active, this technique has a high cell-type specificity.
As the second prerequisite for specific neuronal manipulation in addition to a high degree of spatial resolution, temporal resolution and directional modulation of signaling are required for remotely controlling neuronal firing. Temporal resolution implies the precise control when a receptor or pathway is active or inactive and for how long it should be in a specific active status. Temporal resolution can vary from milliseconds (see 'opsins' described below) to hours (e.g. DREADDs). Important are also 'onset' kinetics (the time between the experimental manipulation and the modulation of the receptor or signaling pathway) and 'offset' kinetics (the time between the initiation of the signaling modulation and the termination of the modulation, Rogan and Roth, 2011). Directional regulation describes the effect of the tool on neuronal activity (either activating or inhibiting), while bidirectional control would be the optimal case: Turning on and off the same cell population would elucidate the full spectrum of function that a cell provides within a particular network for perception or execution of a distinct behavior.

For manipulation of neuronal networks for minutes to hours designer G protein-coupled receptors have been developed. G protein-coupled receptors pathways are involved in a multitude of cellular functions. Unlike opsins, which are functionally silent without excitation in vivo, as they are not directly activated by endogenous compounds, G protein-coupled receptors (GCPRs) are constantly modulated by endogenous ligands in vivo or reveal ligand-independent activity (Seifert and Wenzel-Seifert, 2002; Smith et al., 2007). In vitro and in vivo pharmacological studies have described GPCRs as the most important class of druggable targets in the human genome (Giguere et al., 2014), through which 50% of precribed therapeutics act (Overington et al., 2006). These facts made the development of highly selective orthologous ligand-receptor pairs, which would enable a high spatio-temporal control over GPCR signaling pathways in vivo, challenging (Conklin et al., 2008). In recent years mutations to more than a dozen native GPCRs have opened the field for the development of selectively activated designer receptors. Most of these receptors are divided in two classes: the first -generation RASSLs (receptors activated solely by synthetic ligands) and the second-generation DREADDs (designer receptors exclusively activated by designer drugs), which were evolved through directed molecular evolution in yeast (Rogan and Roth, 2011). RASSLs were first engineered on the basis of serotonin-receptors (Kristiansen et al., 2000), histamine receptors (Bruysters et al., 2005) and melanocortin-4 receptors (Srinivasan et al., 2003). However, these first generation of orthologous ligand-GPCR pairs revealed potential shortcomings: Although the receptors were activated solely by the synthetic ligands, the ligands themselves did not solely activate the designer receptors (as reviewed by Rogan and Roth, 2011). Thus, for the development of second-generation DREADDs Armbruster et al., 2007 took a designer ligand, clozapine-n-oxide (CNO), which was known to be inert at endogenous targets and highly bioavailable and blood-brain-barrier-permeant in both humans and mice (Chang et al., 1998; Weiner et al., 2004). As CNO had a modified structure of clozapine, which was known to be a weak partial agonist at muscarinic receptors, Armbruster et al., 2007 tested mutations induced in the five members of the muscarinic cholinergic receptor family upon their selective responsiveness to CNO application. Introducing two mutations transformed the hM3 receptor into a designer receptor which was insensitive to its native ligands, but highly sensitive to the designer ligand CNO. In smooth muscles cells the G_q-coupled hM3 DREADD receptor stimulated a cascade of inositol phosphate hydrolysis, calcium release and ERK1/2 activation, while the hM4Di DREADD receptor, derived from the G_i-coupled human muscarinic M4 receptor, inhibited forskolin-induced cAMP formation and activation of GIRK causing hyperpolarization and inhibition of neuronal firing (Armbruster et al., 2007).
Since the first development of DREADD receptors reports of their usage in vivo are now appearing: The pharmacokinetic properties of the DREADD ligands and the particular route

of administration (oral administration, subcutaneous, intraperitoneal or even local stereotaxic infusion) determine how quickly neurons response to experimental manipulation by ligand application. Responses typically emerge 5 to 15 min after systematic application e.g. of CNO and usually last for 2h - but this time period can be further enlarged upon dose-dependent increase of CNO (Guettier et al., 2009). When Ferguson et al., 2011 used virus-mediated expression of the hM4Di receptor in the direct and indirect pathway neurons of the striatum they found altered behavioral plasticity associated with repeated drug treatment. In particular decreasing striatopallidal neuronal activity facilitated behavioral sensitization to drug treatment. In another study by Krashes et al., 2011 the activation of agouti-related protein (AgRP) neurons in the hypothalamus by DREADD receptors was used to manipulate feeding behavior, energy expenditure, and ultimately fat stores.

Although manipulation of GPCR signaling pathways by DREADD receptor induction and activation is highly efficient and shows a very specific spatial resolution (depending on the constructs or transgenic mouse lines used), the temporal resolution remains limited to an activation within minutes - due to the slower nature of GPCR signaling and the necessary ligand delivery to the location of neuronal manipulation. In contrast, high-temporal (milliseconds) and cellular precision within intact mammalian neural tissue for fast, specific excitation or inhibition even within a freely moving animal can only be achieved with optogenetics (Fenno et al., 2011).
Early approaches to use light to stimulate neuron activity included the selective photostimulation of neurons in Drosophila by coexpression of the drosophila photoreceptor genes encoding arrestin-2, rhodopsin and the alpha subunit of the cognate heterotrimeric G protein which enabled the sensitization of neurons to light (Zemelman et al., 2002). In a second approach action potentials in hippocampal neurons were induced in a reliable and temporarily precise manner by uncaging a caged capsaicin derivate by light (Zemelman et al., 2003). However, depolarization occured within 5 s after a 1 s light pulse, lasting for 2-3 s and did not attenuate with multiple light pulses. Other approaches such as UV light-isomerizable chemicals linked to genetically encoded channels (Szobota et al., 2007; Gorostiza and Isacoff 2008) had also shown limitations due to reduced speed, targeting, tissue penetration or applicability because of their multicomponent nature (Fenno et al., 2011). In 2003, Nagel et al., cloned channelrhodopsin-2 (ChR2), a cation channel from the green alga Chlamydomonas reinhardtii which depicted similarities to the vertebrate rhodopsin which opens in response to blue light allowing potassium ions to enter the cell. Two years later, the first optogenetic experiment in neuroscience was conducted by expressing ChR2 using a lentiviral vector in cultured rat hippocampal neurons (Boyden et al., 2005). Illumination of these cultures with shorter wavelength blue light (450-490nm) initiated large and rapid depolarization, while light with longer wavelengths (490-510nm) induced smaller currents. Light stimulation of neurons was selective to those neurons expressing ChR2. Since then neuroscientists rapidly adapted the possibilities of this new technology to in vivo experiments. In addition, the palette of available light-sensitive channels and ion pumps for neuronal inhibition and activation, for fast and slow acting opsins and opsins activated at distinct wavelengths has been extensively augmented in recent years (Yizhar et al., 2011; Schmidt et al., 2014; Chuong et al., 2014; Klapoetke et al., 2014).
However, although the numerous advantages of optogenetics are evident - such as the highest specificity, the ultrafast millisecond time scale dissection and basically no adverse effects due to the light (unless the light sourse is not too strong or applied too long) - for many in vivo experiments optogenetics stays an invasive procedure: As the light sourse has to be brought close to the neuronal tissue, targeting deep brain areas or diffuse neuronal populations remains challenging. New opsin developments such as Jaws - an inhibitory opsin, which is activated by light of infrared wavelength (Chuong et al., 2014) - opens up new possibilities for non-invasive manipulation in vivo using optogenetics.
Only very few studies have been applied optogenetics in experimental stroke research so far,

mostly in the context of light-based motor mapping as described above (Harrison et al., 2013). In a first study by Cheng et al., 2014 optogenetic stimulation of the ipsilateral primary motor cortex in ChR2 transgenic mice promoted functional recovery and the induction of growth promoting genes after stroke induction in the striatum and somatosensory cortex.

2.3 State-of the art techniques to combine anatomy and molecular biology

We have described in the last paragraphs how stroke reorganization can be studied on the marcro level of map shifts, as well as by studying single neurons in neuronal circuits using 2-photon calcium imaging approaches. We have discussed how individual neurons and whole neuronal populations can be manipulated with high spatio-temporal resolution disclosing new possibilities of causally linking individual neuronal activity with a distinct behavioral phenotype. What is now missing, is the understanding of the underlying molecular crosstalk inducing anatomical and ultimately behavior changes. Classically, tracing techniques (e.g. dextran tracers) have been applied to visualize cells involved in structural reorganization after stroke (Starkey et al., 2012; Lindau et al., 2014). Li et al., 2010 found a way to exclusively study molecular changes in newly outsprouting neurons ('the sprouting transcriptom') in the peri-infarct cortex by injecting two different fluorescent conjugates of the tracer cholera toxin B (CTB) into forelimb sensorimotor cortex at different times points: One CTB tracer was injected at the time of stroke, the second differently labeled one either 7 or 21 days afterwards. Neurons which expressed only the second tracer were those that had not had an axonal projection to the injection site at the time of the injection of the first tracer, and thus represented neurons which established a new projection pattern after stroke. Both neuron types (single- and double labeled ones) were laser captured to identify the distinct transcriptional profile of an outsprouting neuron in the peri-infarct cortex.
In addition, new constructs are now developed for molecularly profiling projecting neurons and thus bridging the gap between anatomical modification and underlying molecular mechanisms. Using e.g. bacterial artifical chromosome (BAC) transgenic mice which express EGFP-tagged ribosomal protein L10a in defined cell populations allowed purification of polysomal mRNAs from genetically defined cell populations in the brain (Heiman et al., 2008; Doyle et al., 2008). In another study by Ekstrand et al., 2014 ribosomes were tagged with a camelid nanobody raised against GFP enabling the selective capture of translating mRNAs in projecting neurons.

Conclusion

Here we have reviewed current and new promising state-of the art techniques for studying reorganization after stroke, for the identification and manipulation of distinct neuronal populations involved in restoration of function and approaches which allow to examine molecular profiles of neurons which are part of the cortical reorganization process. These techniques open up tremendous possibilities to analyze plastic processes and to target key players for the development of new therapies in stroke.

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Aim of the present work

Only 5% of all stroke patients arrive early enough at hospital to be eligible for thrombolysis therapy increasing their chances for a good recovery. For the other >90% of patients their outcome heavily depends on the size and location of the lesion. Although forms of spontaneous recovery exist, strokes with large lesions, where extensive parts of the sensorimotor cortex are destroyed, reveal only little spontaneous capacities for functional restoration accompanied by poor anatomical plasticity. Rehabilitative therapy, electrical stimulation and growth promoting treatments such as Anti-Nogo A immunotherapy are well known to enhance functional recovery. Experimentally all three treatment options show signs of increased fiber growth and neuronal rewiring in forms of e.g. enhanced numbers of corticospinal fibers sprouting across the midline of the spinal cord from the intact side to the de-nervated one as well as augmented axonal growth of pre-existing ipsilateral projections (Schwab, 2004; Schwab, 2010a; Schwab, 2010b; Dimyan and Cohen, 2011). However, our understand of increased plastic processes in the brain after stroke is still very limited raising also questions about the neurobiology of rehabilitation: How do external interventions such as rehabilitative training or growth-promoting therapies interfer with intrinsic reorganization and rewiring processes? Are there critical, vulnerable time periods for brain repair? Do distinct plastic phases during the reorganization process after stroke correlate with functional outcome? Will we be able to go beyond correlative assumptions and distinguish meaningful newly formed circuits for regain of lost function from spared connections? If we will have closed the loop from anatomical rewiring to behavior outcome will we be able to design better, more optimized rehabilitative schedules?

The present work addresses these questions as follows:
In our first approach (chapter 3) we investigated reorganization in the peri-infarct area after a small stroke destroying the forelimb motor cortex over time in dependence of the restoration of skilled forelimb function. Our aim was here not only to study functional map shifts induced by the stroke but also to analyze neuronal capacities in the peri-infarct cortex to 'take over' function previously represented by now destroyed areas while preserving original functions.
In chapter 4 we studied the combination of Anti-Nogo immunotherapy and rehabilitative training of forelimb function after a large photothrombotic stroke destroying the sensorimotor cortex of the preferred paw. In this experiment we questioned if timing is crucial for functional outcome if two different treatment strategies are either applied concurrently or after each other, whereby each of the approaches alone already enhanced plastic processes. We aimed at not only identifying the optimal rehabilitation schedule but also at exposing the underlying anatomical rewiring mechanisms responsible for the measured outcomes.
In Chapter 5 we investigate if different rehabilitative schedules (such as the rehabilitative paradigms applied in chapter 4) induce distinct spatio-temporal expression patterns of growth-promoting and neurotrophic factors after stroke. These data allow first insights into underlying molecular mechanisms for anatomical rewiring and restoration of function in dependence of external rehabilitative interventions.

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Part II

Results

Chapter 3

Experimental studies to understand reorganization and spontaneous recovery after a small stroke in the forelimb motor cortex

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A.S.W. designed, conducted, analyzed and interpreted the experiments.

Collaborators: C.v.A. provided raw data of behavior and stroke lesion analysis as part of the work for her master thesis supervised by A.S.W. W.O. performed chronic window implantations and provided matlab scripts for analysis of optical mapping. A.S. performed MRI imaging. F.H. and M.E.S. discussed the results and provided technical and financial support.

Abstract

Once a stroke occurs victims suffer from lifelong disabilities including the impairment of speech, vision and motor control. No pharmacological therapy is currently available to stimulate the restoration of function after a large stroke. However, in cases of smaller strokes even without therapeutical interventions a certain degree of spontaneous recovery exists while the underlying principles of increased plasticity promoting map-shifts and rewiring of neuronal circuitry are not well understood. The brain region adjacent to the stroke damage - the penumbra zone - has been shown to be critical for stroke rehabilitation. Thus, understanding the reorganization of the peri-infarct tissue around small cortical strokes over time and its contribution to functional recovery may inaugurate the development of new treatments and improve rehabilitative strategies. Here we used Thy1-ChR2-YFP transgenic mice to initiate a small photothrombotic stroke through a chronically implanted cranial window targeting the forelimb motor cortex. Optical mapping was performed to study the reorganization of the peri-infarct sensori-motor cortex and correlated to the level of regained forelimb function using detailed kinematic analysis. Animals showed constant improvement and complete recovery during 4 weeks after stroke in two grasping tasks, while optical mapping revealed a shift for the center of forelimb function towards the cortical area originally representing the hindlimb. Temporarily and reversibly shutting-off neurons in the peri-infarct area using a virus-based pharmacogenetic approach resulted in a decline of regained motor performance and the re-emergence of motor errors observed initially after stroke onset, indicating the functional relevance of the penumbra for recovery and for the re-establishment of motor engrams involved in skilled forelimb function.

3.1 Introduction

A high degree of spontaneous recovery of function is very often found in small cortical strokes, both in experimental and clinical studies. The mechanisms underlying the recovery are not well understood, but have been attributed to events similar to those involved with plasticity in the intact brain (Kleim and Jones, 2008): Stroke recovery implies both, the structural and functional modifications of brain circuitry which are closely related to the stroke affected area either spatially or functionally or both (Murphy and Corbett, 2009). Two major factors contribute to the increased plastic capacity of the adult brain after stroke: First, a great amount of dispersed and redundant connections between cortical and subcortical regions exists, meaning that if one circuit is destroyed, the next related one is recruited to take over function. Second, new circuits can form through rewiring and remapping of related regions (Murphy and Corbett, 2009).
The brain region adjacent to the stroke damage which typically experiences reduced blood flow - the so-called penumbra - has been often the center of attention, both experimentally and clinically (Rosso and Samson, 2014). Surviving neurons in the penumbra depict enhanced neuroplasticity and undergo active functional remodeling after ischemic insult to form the basis for neurorehabilitation (Feydy et al., 2002; Werhahn et al., 2003; Clarkson et al., 2010; Dancause and Nudo, 2011). In vivo two-photon imaging revealed that dendrites in the penumbra are damaged by stroke but can regain their structure during the restoration of blood flow (Zhang et al., 2005; Li and Murphy 2008). On the molecular level a distinct axonal sprouting transcriptome has been described in the penumbra(Li et al., 2010) which includes the distinct induction of neuronal growth-promoting genes (e.g. neurotrophic factors such as BDNF and Gap-43) in sequential waves (Carmichael et al., 2005). In contrast, growth inhibiting molecules such as chondroitin sulfate proteoglycans are in particularly repressed by the peri-infarct cortex (Carmichael, 2006), while within the glial scar representing the wall which separates the stroke core from the surviving per-infarct tissue growth-inhibiting factors are induced.
Functionally, the ipsilesional cortex has shown increased activity such that representations of impaired body parts shift towards undamaged surrounding areas. This cortical remapping is organized activity dependently, and based on similarity of circuit function as well as competition (Murphy and Corbett, 2009). In particular after a small cortical stroke, recovering per-infarct areas with compromised circuitry compete for map territory with healthy adjacent tissues (Winship and Murphy, 2008; Brown et al., 2009), thus making recovery levels highly dependent on reorganization of peri-infarct tissue with similar function (Cramer et al., 2006). In stroke patients, an enlargement of the cortical hand representation was found to be correlated with improved arm/hand kinematics and clinical scores (Cicinelli et al., 1997, Ward et al., 2006, 2007). In a mouse model, the destruction of the forelimb sensory cortex by a stroke induced new sensory representation in the territory previously only occupied by the forelimb motor cortex (Winship and Murphy, 2008; Brown et al., 2009). In an endothelin-1 rat model which initiates focal strokes, Starkey et al., 2012 found that, when destroying the forelimb motor cortex cells in the hindlimb-sensory-motor area reorganize and become functionally connected to the cervical spinal cord using intracortical microstimulation. However, it is unclear if the primary hindlimb senorimotor cortex maintains its primary role in addition to newly covering for the forelimb motor cortex. Furthermore, it remains elusive, when a map shift occurs, how the lost motor engram due to stroke is re-established within the newly organized sensorimotor representation.

Here we used transgenic channelrhodopsin-2 mice which express a light-sensitive cation channel in layer 5 cortical output neurons (Arenkiel et al., 2007) to perform optical mapping of the sensorimotor cortex. In contrast to the limitations of intracortical electrical stimulation this method allowed us a longitudinal study to examine the cortical reorganization process after a small forelimb stroke. We not only demonstrate the critical role of the peri-infarct cortex for

the functional recovery but also show how the motor engram for fine skilled forelimb function re-emerges in the per-infarct cortex 4 weeks after stroke.

3.2 Material and Methods

Animals

Transgenic Th1-ChR2-YFP mice expressing channelrhodopsin 2 in layer 5 of the sensorimotor cortex (Arenkiel et al., 2007), aged 2 – 4 months, weighed 19-23g, of both sexes were used in this study. Mice were housed in groups of two to six per cage, on a 12h light:dark cycle. Food and water was given ad libitum, except during the training phase and the baseline recordings, when water was removed 4-6 h before the behavioral task in order to use it as a reward during the behavioral conditioning (see 'The conditioned running wheel task' below). All experiments were performed in accordance to ethical guidelines and approved by the veterinary office of the canton of Zurich, Switzerland.

Chronic window implantation over the sensorimotor cortex

For chronic window implantation we adjusted the protocol by Holtmaat et al., 2009 to our conditions: Mice were anesthetized with 3% isoflurane (via a table top mounted anesthesia machine, Tec3 vaporizer, Medical Supplies and Services Int'l Ltd, UK) and head-fixed in a stereotaxic frame. Eyes were covered Vitamin A cream (Braun) for light protection during surgery. The fur above the skull was removed using depilatory cream (Klorane). After a midline incision the skin was retracted and the skull exposed and cleaned. All soft tissue was carefully removed using a sharp blade. We also partly dissolved the fascia of the temporal muscle from the underlying skull to enable a sealing of the dry skull with iBond (iBond self etch; Heraeus Kulzer, Hanau, Germany). We then marked the center of a circular cranial glass window (4mm in diameter) 2 mm lateral to bregma (Tennant et al., 2011, Fig. 1). A ring of Charisma®(Heraeus Kulzer, Hanau, Germany) was applied at the skull indicating the dimensions of dental cement fixation and including the labeling for the glass window. The ring was filled with UV light-cured dental cement (Tetric EvoFlow; Ivoclar Vivadent, Schaan, Liechtenstein. A blue light source ($100mW/cm^2$; Bluephase style, Ivoclar vivadent, Schaan) was used for polymerization of iBond, Charisma®and Tetric EvoFlow. After a craniotomy removing the skull at the labeling for the glass window, the unblemished dura was covered with a circular cover glass and set flush with the skull. The cover glass was sealed with Tetric EvoFlow; Ivoclar Vivadent, Schaan, Liechtenstein. A blue light source ($100mW/cm^2$; Bluephase style, Ivoclar vivadent, Schaan). The skin was attached to the ring of Charisma®with acrylic glue (Histoacryl; Braun, Tuttlingen, Germany) (Fig. 2). For postoperative care all animals received analgetics (Rimadyl, $2.5mg/kg$ body weight, Pfeizer) at least for 3 days and antibiotics (Baytril, $5mg/kg$ body weight, Bayer) for a week. The animals were checked and weighted on a daily basis over the whole experimental period.

Headpost fixation

Following fixation to a stereotactic frame under isoflurane anesthesia the body temperature of the animal was maintained at 37°C. After a midline incision the skin was retracted and the skull was exposed, cleaned and soft tissue and blood remnants were removed. iBond (iBond self etch; Heraeus Kulzer, Hanau, Germany) was applied to the cleaned, nearly dry skull, followed by polymerization with a handheld blue light source ($100mW/cm^2$; Bluephase style, Ivoclar vivadent, Schaan). A custom made aluminium head post (<1g weight, Fig. 2)

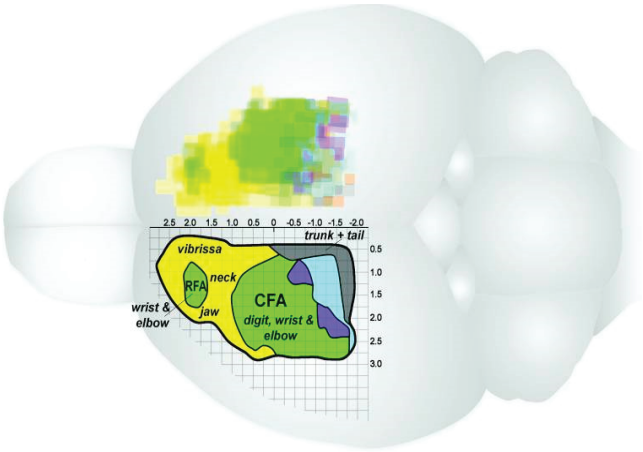

Fig. 1: Organization of the mouse primary motor cortex (M1). A schematic representation of the dorsal surface of the mouse cortex showing movement representation regions simplified by color coding all forelimb-responsive sites as green and head movement representations as yellow. Blue represents movements in the shoulder, while cortical representation of hindlimb movements is color coded in bright blue and grey stands for movements of the trunk. The x-axis representing the horizontal line extending above the map is +2.5 mm anterior and -2.0 mm posterior to bregma (A/P 0.0). The y-axis at the left side of the motor map represents +3.0 mm lateral to bregma (M/L + 3.0). Image adapted from Tennant et al., 2011.

was first glued on the top of the bonding layer of the right hemisphere and then secured by layers of transparent UV light-cured dental cement (Tetric EvoFlow; Ivoclar Vivadent, Schaan, Liechtenstein). The open skin was sutured and attached to the implant with acrylic glue (Histoacryl; Braun, Tuttlingen, Germany).

Photothrombotic stroke

A photothrombotic stroke was induced in six mice to unilaterally lesion the sensorimotor cortex of the left forelimb area (Watson et al., 1985). Briefly, the animal was stereotaxically fixed as described above. The skin was retracted after a midline incision following by labeling of the right motor cortex coordinates (anterior to posterior (AP): -1 to 0.25 mm, medial to lateral (ML): 0 to 3 mm; (Tennant et al., 2011) in relation to Bregma on the skull). The skull was covered with an opaque template (15x15mm, with an opening of 3x1.5mm) and aluminium foil to protect other regions of the brain and the eyes except for the region to be lesioned (left motor cortex, forelimb area). Eight minutes after intraperitoneal (i.p.) injection of 0.1 ml Rose Bengal (10 mg/ml Rose Bengal in 0.9% NaCl solution, Sigma Aldrich, Switzerland), the skull was illuminated by a cold laser light source (Olympus KL1500LCD, 150 W, 300 K). Different illumination times (8-9 min) were applied to find the stroke-size optimum. The wound was then closed and sutured.
For the photothrombotic stroke through the chronic glass window, the rostral half of the window area was covered with aluminium foil sparing the anterior forelimb region. Eight minutes after intraperitoneal injection of the Rose Bengal solution the brain was illuminated for 8 min with the same cold laser light source(Olympus KL1500LCD, 150 W, 300 K).

Asssessment of forelimb function

We used two tasks - the horizontal ladder walk test and the conditioned running wheel task - to train and assess the animals for fine skilled forelimb motor function. Mice received 'baseline' training in both tasks for at least 5 days before stroke surgery, after acclimatization to the testing apparatus in two daily sessions (10 min per mouse). As both tasks contained the precise

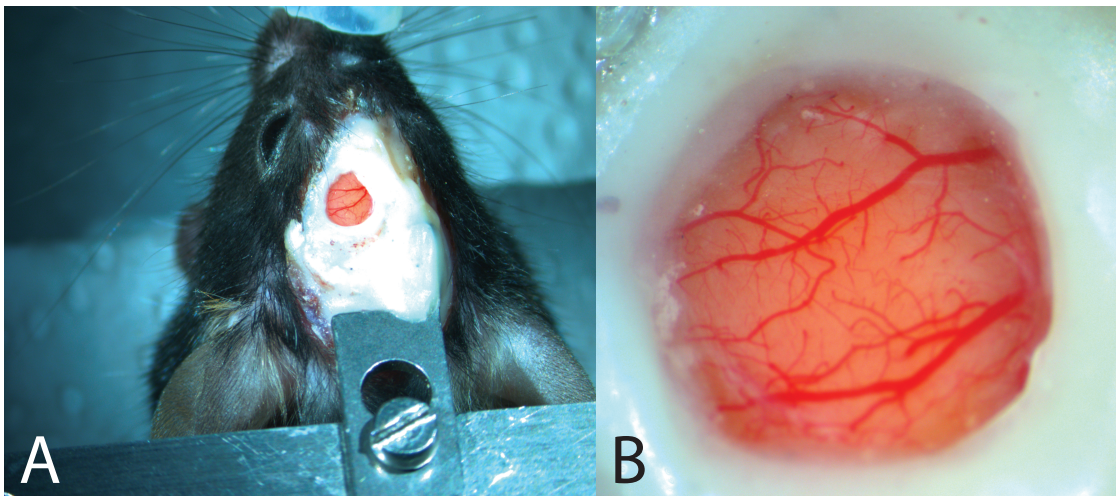

Fig. 2: Chronically implanted glass window for light-based motor mapping and other in-vivo longitudinal imaging studies. (A) Image depicting a mouse with a chronically implanted cranial glass window exposing the sensorimotor cortex. The mouse is headfixed using a screw which anchors the headpost to a metal frame. (B) Cranial glass window displaying the vascular pattern covering the surface of the sensorimotor cortex.

grasping of rungs, the rung sequences were irregularly set to prevent the habituation to the rung pattern and the activation of solely central pattern generators in the spinal cord without requirement of motor-cortical control. For 'baseline' recordings three independent trials were video-taped using high speed camera (high speed camera, Nikon, AF NIKKOR) at a frame rate of 100 Hz. They were then evaluated using frame-by frame video analysis (virtualDub 1.9.11, Avery lee) before stroke onset. Stroke-animals were tested one, three and seven days and then on a weekly basis until four weeks after stroke induction in both tasks, the horizontal ladder and the wheel set-up.

The horizontal ladder walk test

The Horizontal Ladder walk test was used as a method to assess movement coordination and paw placement as an grasping event on the rung. The apparatus consisted of the horizontal ladder construct itself with metal rungs in irregular sequence (distance between rungs 1-3cm) placed between two sidewalls made of plexiglas. Below we placed a mirror in a 45° angle to the camera which enabled us to simultaneous record from two perspectives - from below and from the side view - while the animal was running on the ladder. Each animal crossed the ladder in the same direction three times. For the evaluation of correct paw placement/ grasping events, forelimb footsteps were analyzed using a 4 point rating system as described by Maier et al. 2008. Briefly, a step was counted to be correct when all four digits were placed in front of the rung while enclosing it (4 points). For deficient grasping the following score was applied: (1 point) animals misplaced the digits on the backside of the rung; (2 points) animals placed the wrist instead of the paw on the rung and even slipped off the rung occasionally; (3 points) animals completely missed the rung. Success rates were expressed as a percentage of perfect grasps (perfect grasps/total grasps x 100) and were scored as average of three passes.

The conditioned running wheel task

As a second task for the evaluation of skilled forelimb motor function with an emphasis of skilled grasping abilities we developed the conditioned running wheel task (Fig. 2). Mice were placed on a ultra-light wheel with sidewalls from plexiglas and fiber-reinforced plastic rungs in irregular sequence while being head-fixed by the implanted headpost to a metal rod with a screw. (Fig. 2A). The wheel emulates a horizontal ladder, while keeping the mouse stationary.

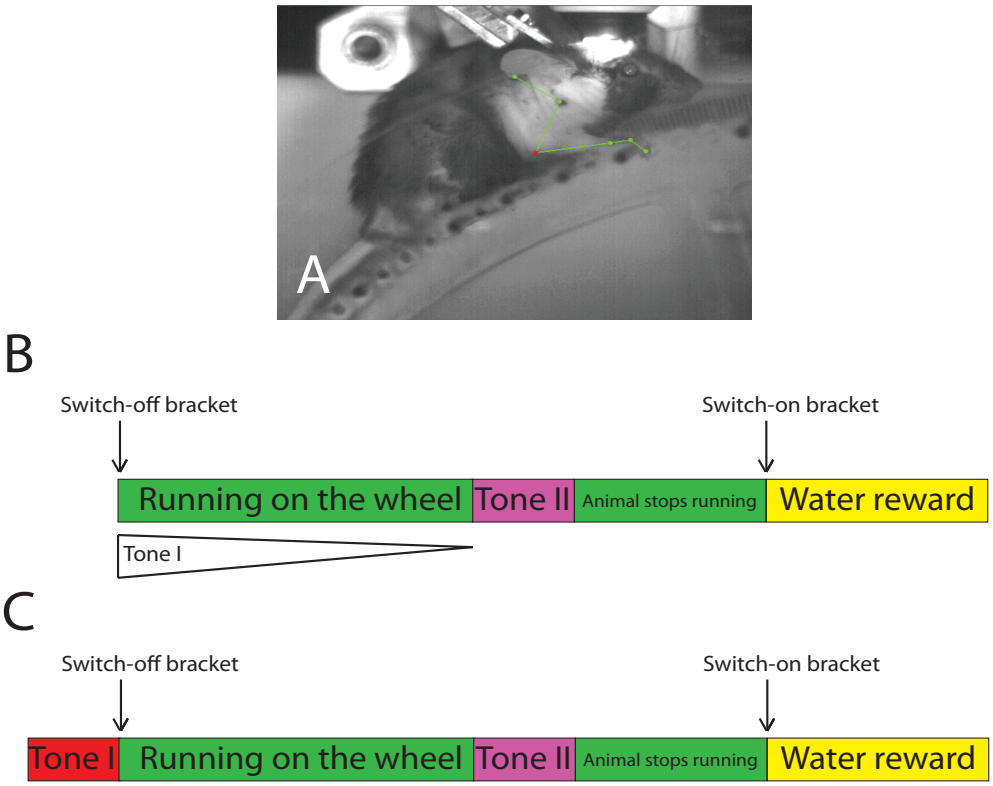

Fig. 3: The conditioned running wheel task. (A) Image depicting a mouse on the running wheel. (B) Scheme I showing the time sequence of the conditioned running wheel task, when the first tone (12000 Hz) decreases in intensity (starting from 80 dB) while the mouse is running on the wheel. (C) Scheme II showing the time sequence of the conditioned running wheel task, when a first high frequency tone (12 000 Hz, 80 dB) marks the switch-off of the brackets allowing the mouse to run till a second tone (4000 Hz, 60 dB) indicates the 'reward phase' of the task, when the water reward is presented.

To augment the motivation of the animals to run on the wheel we established a custom-made conditional reward system with two slightly diverse task set-ups:

(1) Fig. 3B: Animals are exposed to a high pitch start tone (12000 Hz, 80 dB), which would initiate the release of the brakes of the wheel. While the animal is running on the wheel, sound intensity of the high pitch start tone is decreasing until the animal would have circulated a distinct distance (at least half a wheel diameter), as measured by a sensor. Once this set distance is completed a lower pitch reward tone (4000 Hz, 60 dB) is displayed, while the brakes stop the wheel. The mice are rewarded with a drop of water from a water port mounted to a piezo film sensor (MSP1006-ND; Measurement Specialties) which triggers the delivery of water ($5-6$ μl) through a miniature rocker solenoid valve (0127; Buerkert).

(2) Fig. 3C: Animals are exposed to a high pitch start tone (12000 Hz, 80 dB) for 2 sec, which would initiate the release of the wheel brakes and serve as a start signal for running a distinct distance (at least half a wheel diameter). After completion of this distance measured by a sensor, a second low pitch stop and reward tone (4000 Hz, 60 dB)is presented for 2 sec., thereupon the brakes stop the wheel and the animals are rewarded with a drop of water as described above.

One testing session consisted of at least 6-8 conditioned trials (either (1) or (2)) dependent on the distance set to receive at least 50-60 stepping movements which were summarized and counted as one trial for final analysis of success scores.

Detailed grasping analysis

For the detailed analysis of stepping/grasping movement, we developed a scoring method which quantifies every grasp in a 12 point scoring scheme to identify the most frequent foot step error patterns: 'Total miss: grasp too long' represents a grasping event where the rung is missed because the grasping distance was too long. 'Total miss: grasp too short' respectively is a grasping event, where the rung is missed because the grasping distance was too short. 'Partial slip with -wirst or -digit' equalizes that the animal initially correctly placed the paw but then slipped and found again foothold using either the wirst or one of its digits. 'Partial placement with wrist' described the initial placement of the wrist on the rung instead of using the palm of the forepaw. A 'slip after correct placement with forward movement' represents the initial correct placement of the forepaw on the rung, but then the animal slips and loses contact to the rung. 'Weight shift to MCP, to lateral forepaw or to elbow' are terms that describe the aberrant shifting of the weight from the palm of the forepaw in a perfect grasping position to either the metacarpophalangeal (MCP) joints, the lateral forepaw or the elbow.

MRI for stroke volumetry

At the end of the behavioral assessment 4-6 weeks post stroke surgery, animals were first anesthetized with 3% of isoflorane followed by a lethal overdose of pentobarbital (eskonarcon, 300-600 mg/kg bodyweight, i.p., Abbott Laboratories). Shortly, after cessation of respiration, animals were transcardially perfused with ringer's solution (containing 1000,000 IU/ heparin (Roche) and 0.25% $NaNO_2$) followed by 100 ml of a 4% phosphate-buffered paraformaldehyde solution (PFA), pH 7.4, to fix the brain. Brains were then removed and placed in PFA (Sigma) solution for postfixation at 4°C.Prior to ex vivo imaging for determination of lesion size the brain tissue samples were placed in 15 ml falcon tubes filled with perfluoropolyether (Fomblin®Y-LC 80, Solvay Solexis, Bollate, Italy). By applying a 7T small animal MR system (Bruker BioSpin GmbH, Ettlingen, Germany), the brain MR measurements were accomplished using a volume resonator for excitation and a four-element phased array surface coil for signal detection. 17 coronary slices of 0.3 mm thickness with an interslice distance of 0.3 mm (no gap between slices) resulted in T2-weighted images which were acquired using a TurboRARE sequence with the following parameter: matrix dimension= 200 x 200, spatial resolution= 50um x 50um voxels, mean diffusivity= 200 x 140,repetition time= 350 s, echo time=10um, number of averages= 120.
After ex vivo MRI, brains were removed from Fromblin and again placed in PFA. The quantification of the lesion volume was performed with Image J, counting the pixels in the encircled area of interest. We first measured volume of both hemispheres separately and then extrapolated the pixel count for the stroke volume to the corresponding volume of the unaffected contralesional hemisphere taking the shrinkage of stroke scar tissue into account.

Histological analysis of stroke lesion

After perfusion and removal, brains were left in 4% PFA overnight and put in 30% sucrose for 3 days. The brains were embedded in Tissu-Tek®O.C.T.TM and frozen in isopentane (Sigma) at −40°C. Brains were cryosectioned (40μm slice thickness) and kept at −20°C. The frozen sections acclimatized to room temperature for 20min before the staining. Brain slices were then washed in H_20 followed by a short incubation time in 0.5% Cressyl violet (2-3 min) for the Nissl staining. After dehydrating in graded alcohol solutions starting with 70% alcohol up to 100%, the sections were cleared in xylene and cover-slipped with Eukit mounting medium. The lesion volume of each brain was evaluated by reconstruction of every 9th brain section, using Neurolucida 8.0 (MicroBrightField).

Light-based motor mapping and map analysis

In order to study reorganization in the left peri-infarct sensorimotor cortex over the course of 4 weeks after stroke we performed light-based motor mapping in the Th1-ChR2-YFP mice, a technology which has been described in detail by Ayling et al., 2009. We used a 473 nm laser beam (Micron laser technology, focused to 100 μm diameter, 10 ms pulses, 2-4 mW total) which was optically deflected by a motorized mirror system controlled by custom made software (Labview, National Instruments) to an array of cortical sites (10 x 10, with 300 μm spacing between sites) covering the whole aperture of the chronically implanted glass window over the sensorimotor cortex (Fig. 4).
Before the motor mapping we applied a mild depilatory cream (Klorane) to remove the fur at the limbs and tattooed small dots at different landmarks to label the joints using black tattooing solution. We marked the following limb structures: the scapula, shoulder, wrist, metacarpophalangeal joint (MCP), digit-tip, hip, knee, angle, metatarsophalangeal joint (CTP) and toe-tip. Before motor mapping mice were anesthetized by a single i.p. injection of ketamine (100mg/kg body weight) followed by another ketamine supplement (30mg/kg body weight) if necessary to complete the mapping. Mice were placed in a hammock (Fig. 4) that enable unrestricted limb movement due to optical stimulation. While the sensorimotor cortex was stimulated forelimb and hindlimb movements were recorded by a video camera synchronized to the mapping controller software. Stimulation was delivered in a semi-random order with identical stimulus intensity for all cortical stimulation sites within a map, with the requisition that sites within 750 μm apart from each other could not be stimulated consecutively. An overlay function within our software allowed us to superpose images of the mapping position from previous mapping sessions to the current one enabling us to use landmarks within the cranial window such as the blood vessel pattern or the cement sealing as reference points to track map positions over time and relate motor maps.
For motor mapping analysis we used a semi-automated motion tracking software (Clickjoint, Aleaso) which tracked the joint markers of the mice frame by frame from the videos, recording the movements of the animal during motor mapping. This approach allowed us to generate two dimensional coordinates (x,y) for every marker and time point. For the kinematic analysis all movements were reconstructed from changes in the marker location between successive frames. The software directly calculated angles and distances and data were then imported into Microsoft Office Excel 2010. We then used a matlab script to create heatmaps depicting the joint angle changes in summary or individualized for each joint in dependence of the cortical stimulation site.

Pharmacogenetic inhibition of neurons in the stroke penumbra

Aiming at testing the functional relevance of the re-organized peri-infarct cortex for the regained forelimb function 4 weeks after stroke the following experimental set-up was applied: Animals (n=6) were pre-trained in the conditioned running wheel task (as described above)and received a photothrombotic stroke impairing the left forelimb motor cortex. We assessed recovery of forelimb motor function over the course of 4 weeks after stroke proceeded by virus injection for short-term reversible pharmacogenetic inhibition of neurons and their connections in the peri-infarct sensorimotor area using the virus mediated expression of an engineered G_i- protein-coupled receptor ($G_{i/o}$-coupled human muscarinic M4 designer receptor exclusively activated by a designer drug, hM4Di).
The surgical procedure was as follows: A craniotomy in the anesthetized (3% isofluran), and stereotaxically fixed mice was performed exposing the left contralesional motor cortex. All animals were injected in the penumbra area 4 weeks after stroke with a mixture of AAV2.1/2.2-

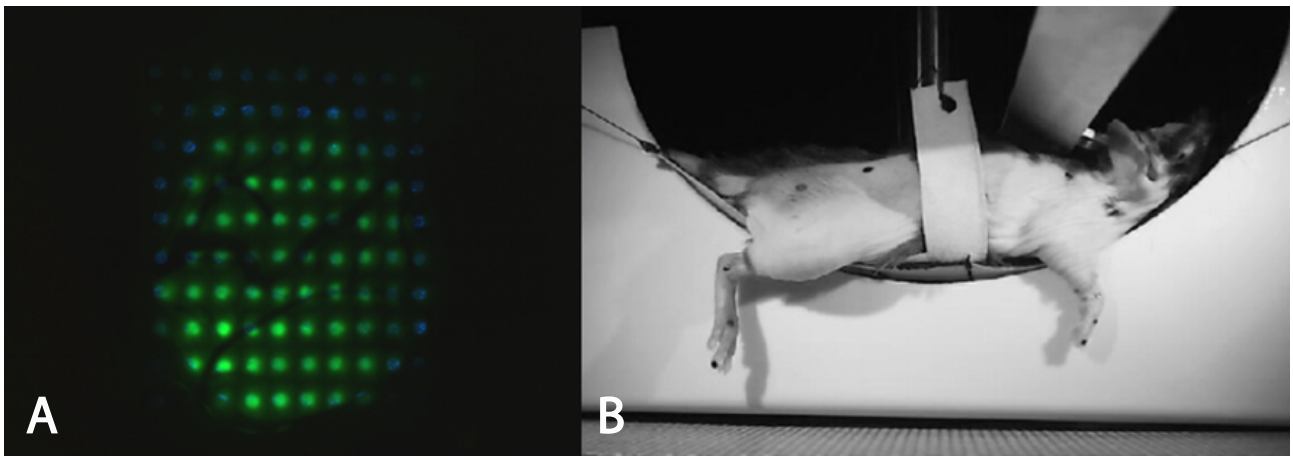

Fig. 4: Light-based mapping of the motor cortex to study reorganization after stroke. (A) Image showing the array of cortical stimulation sites (10 x 10, with 300 μm spacing between sites) covering the whole aperture of the chronically implanted glass window over the sensorimotor cortex. The image is a maximum projection of the sequence of images taken by a CCD camera mounted on top of the microscope during the recording of the sequential arrangement of laser stimuli (473 nm laser beam, 100 μm diameter, 10 ms pulses) at random stimulation sites within the 10 x 10 stimulation grid in a mapping session. (B) Photograph depicting a mouse with tattoos marking the joints of the fore- and hindlimb laying in a hammock which enables free passive movements of the limbs during the light-based mapping session of the sensorimotor cortex.

hSyn-dio-hM4D(G_i)mCherry vector (1:2, UNC Vectore Core, the University of North Carolina at Chapel Hill). The injection sites for the penumbra area were 2 AP 2 ML; 1 AP 2 ML; 0 AP 2 ML; -1AP 1 ML; -2 AP 1 ML with variations among the individual mice. Coordinates were slightly adjusted to the penumbra areas according to the extent of the individual strokes. Injections were made in a coronal manner in at least 0.5 mm distance to the borders of the stroke scar formation in. All stereotaxic injections (2x 20 nl each) were made through the intact Dura using a 35 gauge, 10 μl syringe (World Precision instrument with a flow rate of 6nl/s), controlled by an electrical pump. At each position two injections were performed: the first one at -1mm depth keeping the syringe in place for 1 min followed by a second injection at -0.8mm depth. After the second injection the syringe was left in place for 3 min to allow diffusion of the virus, before the suringe was retracted.

Animals were allowed to recover from the virus injection surgery at least 2 weeks before re-assessment of skilled forelimb function on the conditioned running wheel for at least 3 times and subsequent initialization of the DREADD experiment: Animals were recorded while running on the wheel at 'baseline' (6-7 weeks after stroke surgery) followed by i.p. injection of clozapine-n-oxide (CNO, 5-10mg/kg body weight; Enzo Life Sciences). Animals were put back on the running wheel and stepping performance was recorded 30 min and 2h after CNO injection for further analysis comparing pre-stroke 'baseline' with the post-stroke performance as well as each post stroke grasp with the performance at 30 min after CNO application. The experiment was repeated at least 3 times at independent days. In a control experiment, repeated twice, NaCl was applied instead of CNO to preclude an effect of the injection and treatment that might have influenced the behavior.

At the end of the experiment animals were anaesthetized and transcardially perfused as described above (section 'MRI images for stroke volumetry'). Coronar cortex sections ($40\mu m$ slice thickness) were examined for the distribution of mCherry-positive neurons with anti-mCherry immunohistochemistry: Immediately after cryo-sectioning slices were blocked in Tris-NaCl-blocking buffer and 0.1% Triton (TNB, TBST) for 1h at room temperature and incubated over night at 4°C in TNB, TBST and the primary antibody mCherry (Abcam, Lucernachem). After washing with 0.1 PB, the biotinylated Goat Anti-Rabbit immunoglobulin-G (Ig G) (1:300; Jackson IR) secondary antibody in PBS was applied for 2 h at room temperature. The sections were

washed in PBS and incubated in Alexa 488 Streptavidin (1:1,000; Jackson IR) and fluorescent Nissl 640/660 (1:500; Neurotrace Invitrogen) in TNB for 30-60 min. Slices were washed for the final step. Dried slices were covered with moviol. Images of mCherry positive cells were taken by a confocal microscope (Leica TCS SP2) with red (TRITC) and infrared (Cy5) excitation or emission filters at 20x magnification. mCherry positive cells were counted in relation to Nissl stain positive cells in layer 5 of the sensorimotor forelimb cortex. The count was repeated on 3 sections of each brain. Neurolucida 8.0 was used, taking every 9th section($40\mu m$), to perform 3D reconstruction of brains containing the DREADD virus and to evaluate the localization and expansion of mCherry positive cells in the brain in distance from the stroke lesion.

Statistical analysis

The statistical analysis was performed using GraphPad Prism 6 (version 6.01-Windows; GraphPad Software), Matlab and SPSS. All data are expressed as mean ± standard error of the means (s.e.m.). Whenever two groups were compared e.g. for the detailed paw scoring method before and after a lesion, the student' s t-test (two tailed, unpaired) was used. For the correlation study between the stroke volumetry and the behavioral deficit spearman correlation was applied. For the behavioral data obtained from mice for the spontaneous recovery after unilateral stroke one-way repeated ANOVA measures followed by post hoc Bonferroni tests were used to assess locomotion deficits (baseline vs. 1, 3 days or 1 day after injury vs.14, 21, 28 days). The same test was used for the animals receiving the DREADD virus to evaluate their performance on the wheel before and after CNO application. Two-way ANOVA with post hoc Bonferroni was applied if different error grasping patterns on different time points where compared, as tested for the naïve, the impaired, the recovered and the mice injected with the DREADD virus.

3.3 Results

3.3.1 Establishment of a small forelimb stroke through a chronically implanted glass window

Our approach was to study reorganization in the perilesional sensorimotor cortex after a small stroke affecting the forelimb area in the same animal over the course of weeks with the aim to correlate cortical rearrangement processes with functional recovery. We used transgenic channelrhodopsin-2 mice (Arenkiel et al., 2007) which express a light-sensitive cation channel in layer 5 cortical output neurons to perform longitudinal light-based mapping of the motor cortex as described by Ayling et al., 2009 and Harrison et al., 2013. We first implanted glass windows covering the sensorimotor cortex of the left hemisphere(4mm diameter, expanding 4mm lateral, 2mm anterior and 2mm posterior to bregma) in five mice. After training them in two grasping tasks (horizontal ladder walk test and conditioned wheel running test, Fig. 5B, C) 2-3 weeks post surgery (Fig. 5A) for three independent baseline recordings, animals received a small photothrombotic stroke impairing the forelimb area (Fig. 6).
Our aim was to establish a stroke lesion through a glass window which would be specific and severe enough to target skilled forelimb function but would also be small enough to induce perilesional sprouting and reorganization which we would then study through the cranial window. For that, we applied an opaque template (with central opening of 3x1.5 mm) to the skull of the animals and exposed it to light for 8 min. We found that small strokes of $1.2\pm0.1 mm^3$ (Fig. 5F) of lesion size resulted in a substantial decrease of forelimb function in both grasping tasks up to 7 days after stroke (horizontal ladder task, 39.4 ± 6.0% of baseline function on day 1 after stroke, Fig. 5D; conditioned wheel running task 54.7 ± 5.8% of baseline function on day

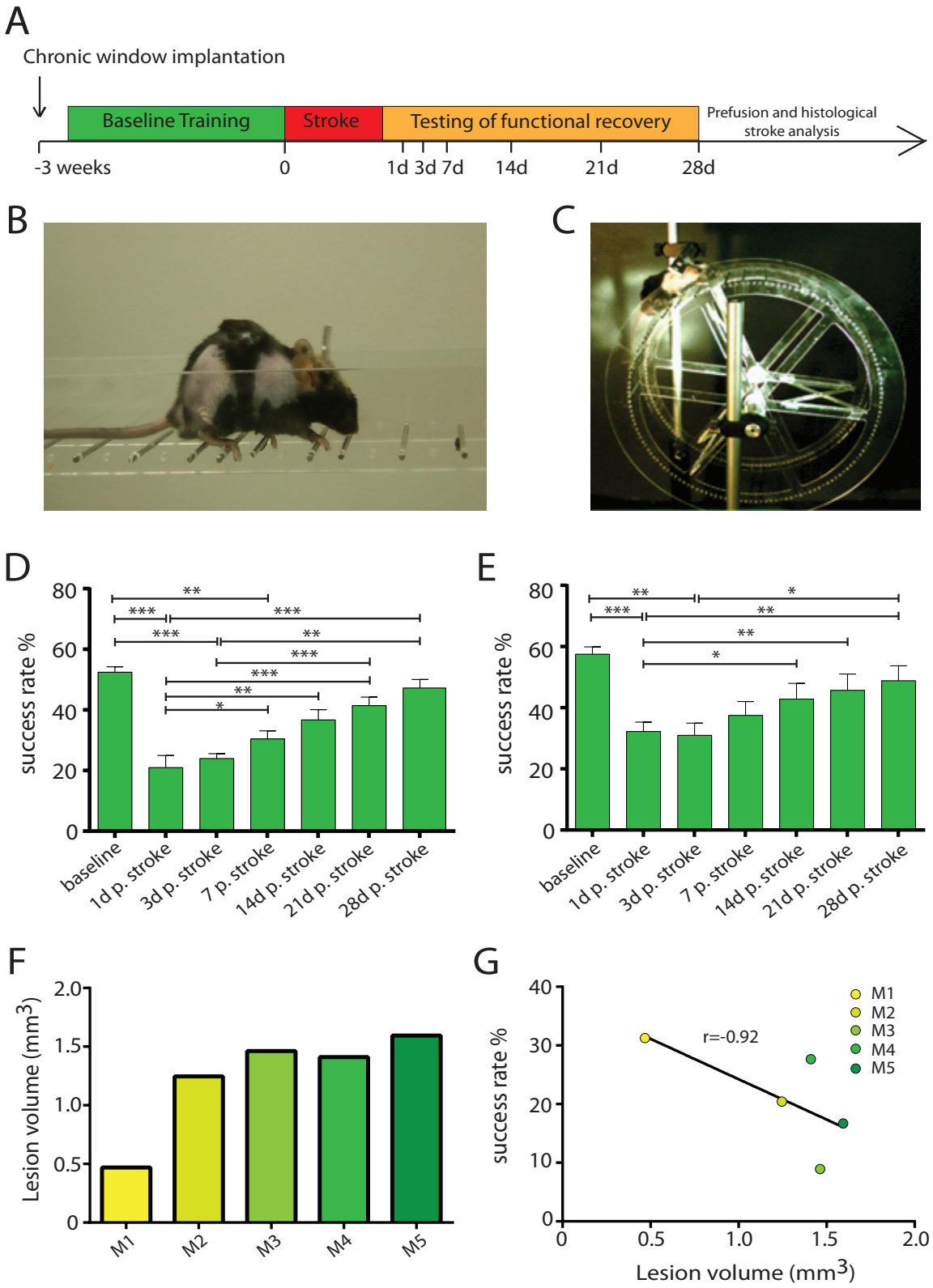

Fig. 5: Mice show full spontaneous recovery of skilled forelimb function 28 days following a photothrombotic stroke destroying the forelimb motor cortex. (A) Experimental time schedule. We used two tests for the assessment of fine skilled motor function of the forelimbs: (B) The horizontal ladder walk test and (C) the conditioned running wheel task. (D, E) 1-3 days post stoke surgery animals (n=5) revealed a significant decline in grasping performance in both tasks compared to baseline sucess scores before stroke. At 28 days after stroke animals gained almost the same levels of success rates than at the healthy baseline condition indicating a full spontaneous recovery of forelimb function. Data are presented as means ± s.e.m.; statistical evaluation was carried out with one-way ANOVA repeated measure followed by Bonferroni post hoc, asterisks indicate significance: $^{*}P < 0.05$,$^{**}P < 0.01$, $^{***}P < 0.001$. (D) Ex vivo magnetic resonance imaging (MRI, 7T small animal MR system) was used for anaysis of stroke volumes in the different animals of this study cohort (M=mouse).(G) There was a correlation between lesion volume and motor impairment at day 1 after stroke (p=0.0227, r=-0.9284, Pearson correlation, M=mouse 1-5).

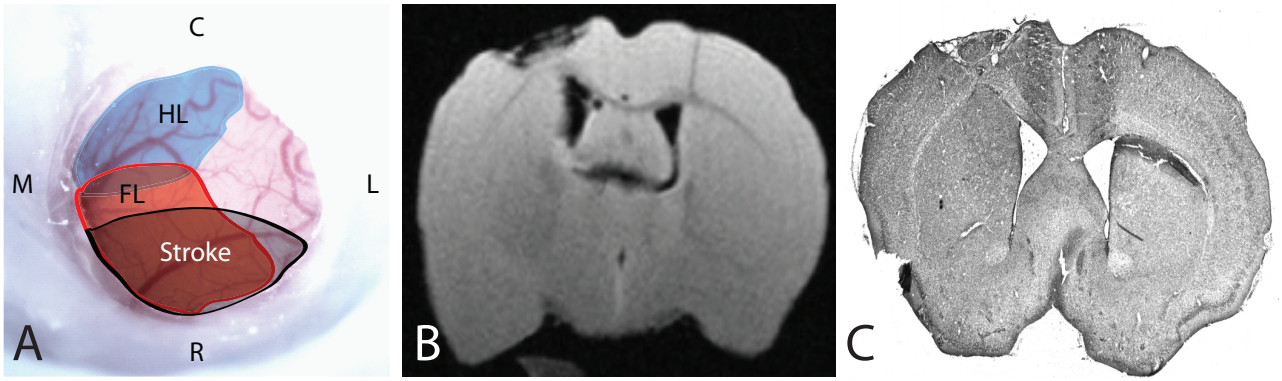

Fig. 6: Establishment of a small photothrombotic stroke destroying the forelimb motor cortex. (A) Scheme showing a cranial window exposing the sensorimotor cortex. Light-based optical mapping was used to identify the forelimb (FL) and hindlimb (HL) motor cortex before a photothrombotic stroke was introduced targeting 2/3 of the forelimb motor cortex from rostral (R= rostral, C= caudal, M=vmedial, L=vlateral relative to bregma). (B) Ex vivo magnetic resonance imaging was used to evaluate lesion size and localization. (C) Representative Nissl-stained section for histological analysis of stroke lesion size in the left forelimb sensorimotor cortex 4-5 weeks post stroke after the completion of the behavioural assessment.

1 after stroke, Fig. 5E, one-way ANOVA, repeated measures). Animals showed spontaneous restoration of forelimb function to a degree of almost full recovery in both grasping tasks (horizontal ladder task, 97.7 ± 6.8% of baseline function on day 28 after stroke, Fig. 5D; conditioned wheel running task, 92.3 ± 3.3% of baseline function on day 28 after stroke, one-way ANOVA, repeated measures). There was also a correlation between lesion volume and motor impairment on day 1 post-stroke (p=0.0227, r=-0.9284, Pearson correlation, Fig. 5G).

3.3.2 Stroke induces a stereotypic error pattern of forelimb movements for grasping

To reveal distinct post injury behavioral failure and compensation patterns of forelimb usage, which are crucial for spontaneous motor recovery analysis we developped a 12 point evaluation score for detecting stereotypical error movement patterns which emerge after stroke onset (Fig. 7) based on the Whishaw Reaching Test assessment scale (Whishaw et al., 2008). We evaluated the grasping distance (if the grasp was too long or too short relative to the rung target), the targeting of the rung (correct targeting, partial slip or miss with either digit, paw or wrist), placement of the forelimb on the rung as well as the balance and weight distribution on the rung and during the grasping/stepping moment(e.g. lateral weight shift, aberrant weight shift to MCP, lateral forepaw and wrist). We then compared the evaluation scores at 'baseline' recordings to those obtained after stroke (1-3d post stoke surgery).
We identified two typical deficit movements which were repetitively detected one to three days post-stroke: The first movement pattern was over-extensive grasping (totally missing the rung) (18.6 ± 3.31% of total grasps analyzed for the time point '1-3d post stroke', $p < 0.001$, Student's t-test, n=5, Fig. 7B) and partial slipping with the wrist (21.68 ± 2.99% of total analyzed grasps 1-3d post stroke, $p < 0.01$, Student's t- test, Fig. 7B). These two types of grasping errors occurred 3-7 times more after stroke than in the healthy animals on baseline recordings: Too long grasps were detectable in 19.5 ± 3.3% of all grasps 1-3d after stroke compared to 5.8 ± 1.8% at baseline while partial slipping after shoring up with the wrist on the rung was measured in 22.7 ± 3.5% of all grasps 1-3d after stroke versus 3.4 ± 1.2% at baseline. Both grasping error sub-patterns also emerged more often than the other error types measured with our classification system (Fig. 7B).

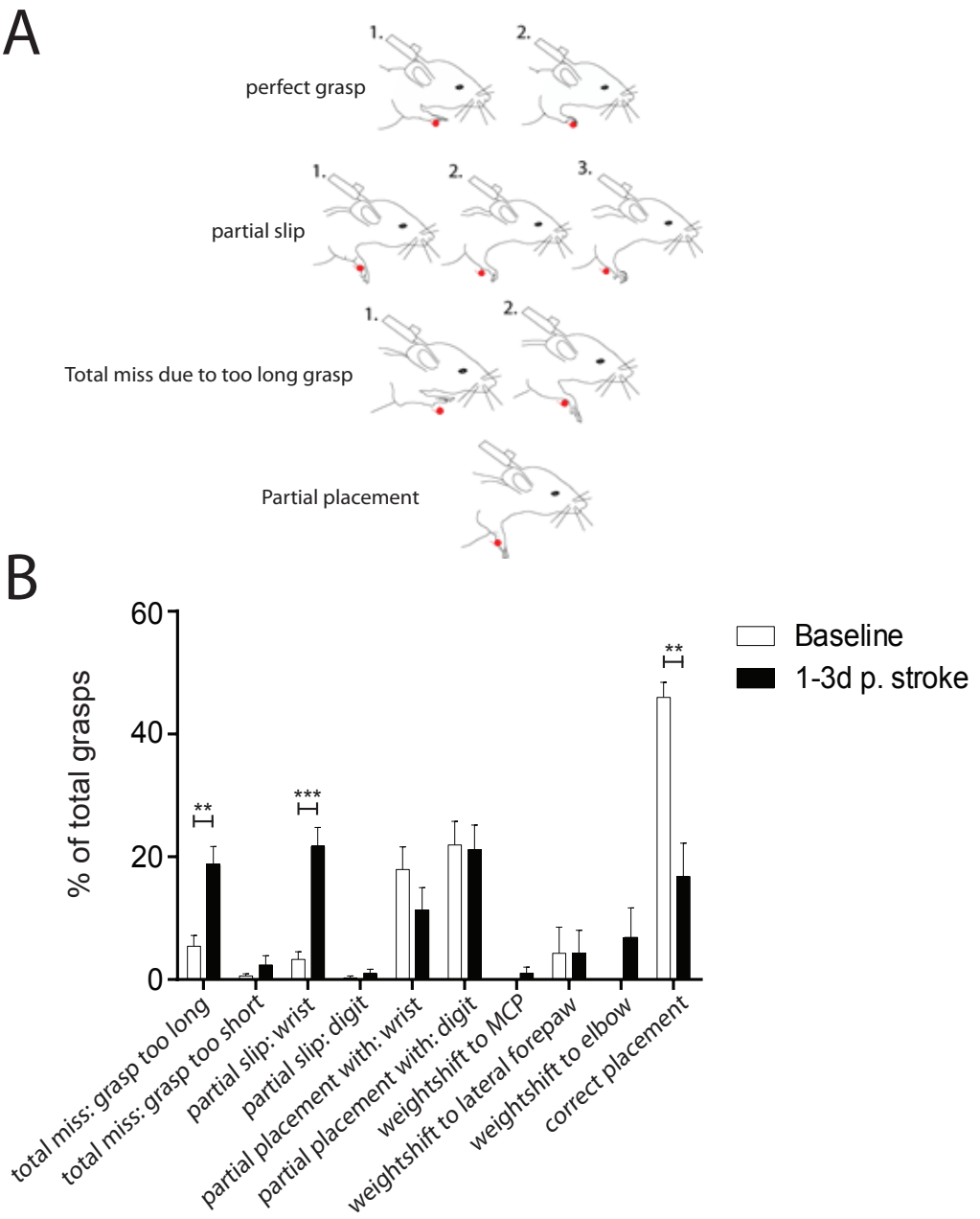

Fig. 7: Stroke induces a stereotypic error pattern of forelimb movements for grasping. (A) Illustration of four stereotypical sequences of movements leading to grasping events which we evaluated before and after stroke to identify and analyze pathological grasping kinematics (adapted from C. v. A.'s master thesis) (B) A detailed dissection of movements leading to grasping features revealed two stereotypical error patterns repeatedly emerging in all mice analysed (n=5) 1-3 days after stroke: The percentage of grasps which failed either because the reaching action was too long, or because the rung was targeted by the wrist joint leading into secondary slipping of the paw, was significant higher 1-3 days post stroke than at the baseline recordings before stroke. Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-tailed Student's t-test, paired, asterisks indicate significance: $^{**}P < 0.01$, $^{***}P < 0.001$.

3.3.3 Center of the forelimb area shifts towards the hindlimb motor cortex during 4 weeks post stroke.

We performed light-based mapping of the sensorimotor cortex (10 x 10 stimulation points covering the full expansion of the cranial glass window, Fig. 8A) while stimulus-evoked movements were video-recorded and angle modifications measured by tracking of limb trajectories (see Material and Methods, Fig. 8B, C) were used as a read-out to generate maps. To obtain a mean value for each pixel of the map three independent baseline motor mapping sessions were executed. 1-3 days after the third baseline session a photothrombotic stroke was targeted to the forelimb motor cortex (Fig. 6) based on the map created as an average of the three baseline sessions revealing the center of the fore- and hindlimb motor cortex (Fig. 8D, E). 2d, 16d and 23d post stroke we stimulated the same cortical sites to study map shifts during the phase of cortical reorganization. While we found no stimulus- evoked movements and thus joint angle changes for the forelimb 2d after stroke (Fig. 8D), a diffuse pattern of stimulus-evoked forelimb movements was detectable among stimulation of a broad range of sensorimotor cortex sites. A map shift for the center of the newly reorganizing forelimb towards the hindlimb area became evident which was further consolidated 23d post stroke (Fig. 8D).In contrast, hindlimb responses were recorded within the same area at baseline and 2d post stroke indicating an exclusive injury of the forelimb cortex by the stroke (Fig. 8E). In the phase of the reorganizing forelimb cortex 16d-23d after ischemic insult stimulus-evoked movement responses notably increased and the cortical hindlimb representation extensively expanded (Fig. 8E). Analysis of cortical motor maps in detail for extension or flexion movements in distinct forelimb joints (shoulder, elbow, wrist and metacarpophalangeal (MCP)) revealed a nearly complete restoration of the center of response for the MCP joint (Fig. 9A versus Fig. 9D for MCP) while we still measured diffuse and disorganized responsive movements in the wrist among several stimulation sites which had been attributed to both fore- and hindlimb cortex at baseline mapping sessions, even 21d after stroke onset (Fig. 9A versus Fig. 9C, D for wrist).

3.3.4 The peri-infarct area is crucial for the restoration of skilled forelimb function after stroke

As we detected a reorganization of the forelimb area with a map shift towards the hindlimb cortex accompanied with an almost full restoration of the cortical representation for the MCP joint, we aimed at closing the loop between cortical reorganization and restoration of skilled forelimb movements: Thus, we used a pharmacogenetic approach to reversibly and temporarily silence peri-infarct neurons in layer 5 of the sensorimotor cortex (Fig. 10B). After pre-training of animals on the horizontal ladder walk test and the conditioned running wheel task, we initiated a photothrombotic stroke destroying the left forelimb cortex (Fig. 10A). 4 weeks post stroke animals revealed nearly full functional recovery in these fine skilled motor tasks (conditioned running wheel task, 96.9 $\pm$ 1.3% 4 weeks after stroke compared to pre-stroke performance) as seen before (Section 'Establishment of a small forelimb stroke through a chronically implanted glass window'). We then injected an engineered Gi-coupled DREADD (hM4D(G_i)) receptor ('designer receptors exclusively activated by designer drugs') at 5 injection sites in the peri-infarct area with at least 0.5 mm distance to the borders of the stroke scar formation. Such receptors are only activated by the pharmacologically inert synthetic ligand clozapine n-oxide (CNO), leading to increased intracellular mediated G-mediated signaling, resulting in hyperpolarization of infected cells and their silencing (Armbruster et al., 2007; Conklin et al., 2008). To test the inhibiting effect of such a pharmacogenetic tool we recorded and analyzed the right-sided forelimb motor performance of the animals before CNO injection for baseline recording (7 weeks after stroke surgery), 30 min, 1 hour and 2 hours following intraperitoneal CNO ap-

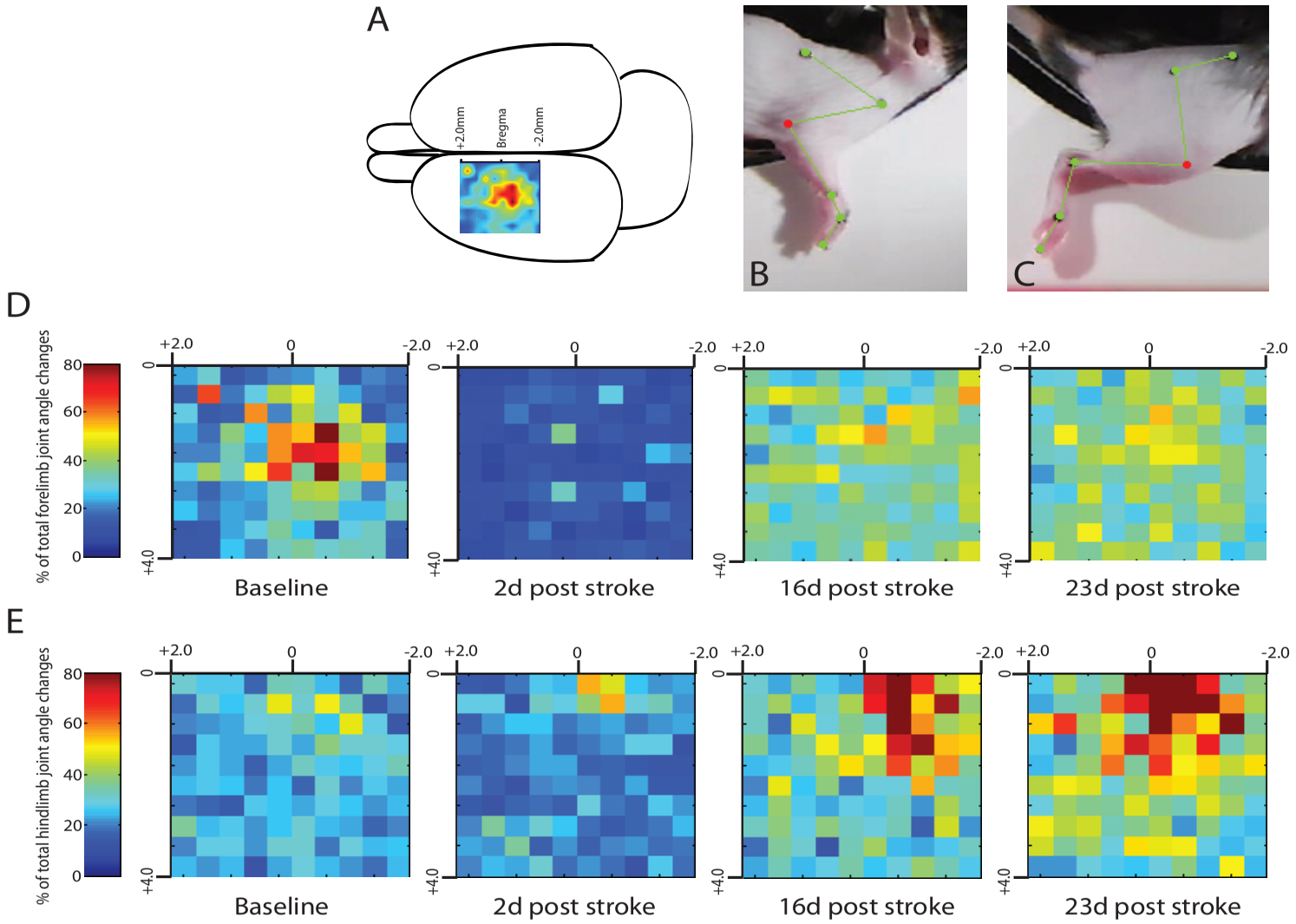

Fig. 8: A new center for forelimb function is organized within a part of the former hindlimb motor cortex after destroying the forelimb area by stroke. (A) Scheme illustrating the position of the 10 x 10 stimulation grid for light-based mapping of the sensorimotor cortex in Th1-ChR2-YFP transgenic mice. (B, C) Photographs showing the trajectories of forelimb joints (B, shoulder, elbow, wrist and MCP, digit) and hindlimb joints (C, hip, knee, ankle, MTP and digit) allowing to detect joint angle changes which were used as a readout for stimulus evoked movement responses upon light-based cortical stimulation. (D) Longitudinal light-based mapping of the sensorimotor cortex reveals the position of the center for forelimb function at 'baseline', which is destroyed by the photothrombotic stroke indicated by the mapping '2d post stroke'. Within 16 days after stroke a diffuse stimulus-evoked response pattern for forelimb movements is detected throughout the sensorimotor cortex which consecutively crystallizes after '23d post stroke' to form a new center for forelimb function within a part of the former hindlimb motor cortex. (E) The structure of the hindlimb motor cortex is preserved at '2d post stroke' compared to the 'baseline' mapping. While parts of the hindlimb motor cortex 'take over' forelimb function, the hindlimb area expands in combination with increased stimulus-evoked movement responses 23d post stroke. The heat maps shown in D and E depict the sum of either all forelimb joint angle changes (D, shoulder, elbow, wrsit, MCP joint) or all hindlimb joint angle changes (E, hip, knee, ankle, MTP joint) per cortical stimulation site (± 2mm anterior/poster and +4mm lateral relative to bregma (A/P, L 0.0) at the indicated time points pre- and post stroke.

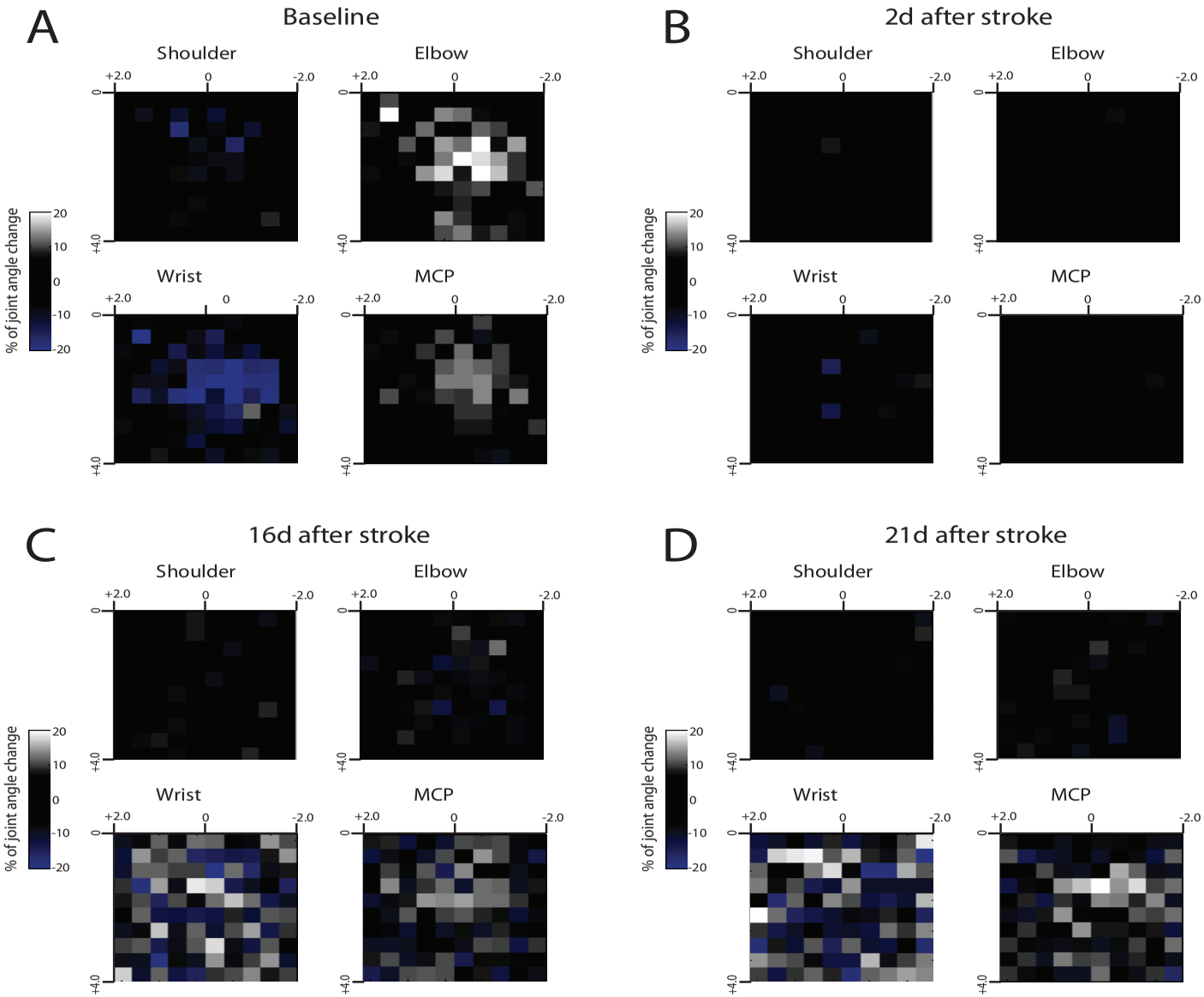

Fig. 9: Cortical map representation for distinct forelimb joints reveals an asymmetric reorganization after stroke. While baseline mappings show distinct cortical representation sites for the four forelimb joints (A), no stimulus-evoked movements in any of the forelimb joints can be detected upon cortical stimulation 2d after stroke (B). While there is almost no reorganization for the elbow joint even 23d after stroke, cortical representation centers re-emerge for the wrist and MCP joints (C, D). The cortical representation for the wrist remains dispersed between 16d and 23d after stroke. In contrast, a map shift for the cortical representation center of the MCP joint is detectable which reorganizes in a part of the former hindlimb motor cortex. The heat maps shown in A-D depict joint angle changes for the four forelimb (shoulder, elbow, wrsit, MCP joint) per cortical stimulation site (± 2mm anterior/poster and +4mm lateral relative to bregma (A/P, L 0.0) at the indicated time points pre- and post stroke. The color code illustrates extensions of joints on the bluish spectrum (0 - (-20)), while joint flexions are colored in the whitish spectrum (0 - (+20)).

plication (Fig. 10A, C). We found that the activation of the hM4Di DREADD receptors in peri-infarct neurons by CNO resulted in a significant decline of forelimb motor performance 30 min after CNO injection in both tasks for skilled forelimb function(for the conditioned running wheel task: 29.8 ± 5.6% of the success rate at baseline recordings before CNO application, Fig. 10C; for the horizontal ladder walk test: 38.4 ± 2.0%, Fig. 10D, two-way ANOVA repeated measures, followed by Bonferroni post hoc test, Fig. 10C). In comparison, in the same animals the induction of the photothrombotic stroke itself disturbed skilled forelimb function with a decrease in the success rate of 39.6 ± 9.9% (Fig. 10C) relative to pre-stroke baseline recordings. The functional defects due to CNO application were fully reversible, with performance returning to pre-injection levels about 1-2h after drug injection in both grasping tasks. In order to exclude an unspecific effect of the drug application or other procedural co-founders, CNO was substituted by i.p. NaCl injection in the same animals and the DREADD experiment was repeated twice. We did not measure any decline of forelimb motor performance after NaCl application in neither of the tests (Fig. 10C, D), indicating a specific inhibition of peri-infarct neurons containing the hM4Di DREADD receptor due to CNO injection.

The DREADD receptor hM4Di was tagged with mCherry, which allowed neuroanatomical localization of the neurons carring the hM4Di vector. Two-dimensional representations of 3D reconstructions revealed the distribution of mCherry positive cells in layer 5 of the peri-infarct area around the stroke area. 42.8 ± 1.6% of Nissle positive cells in layer five of the penumbra region (around 0.5 mm distance to the border of the stroke scar) were also mCherry positive (Fig. 10E, G). Notably, only a very limited number of cells were outside of this area, however the distribution and the amount varied among different animals (Fig. 11), due to slight differences in the stroke expansion and location. We found no significant correlation between the amount of positive cells, expressing the hM4Di vector and the motor performance decline 30 min after CNO injection. This result is most probably due to the inter-individual variability and the small number of animals assessed. However, we could observe a tendency of an augmented decline in motor performance in line with a higher amount of mCherry positive cells in the penumbra (data not shown).

We also used our 12 point evaluation score, which we had developed for detection of stereotypical error movement patterns (as described in section 'Stroke induces a stereotypic error pattern of forelimb movements for grasping'), for the analysis of modified grasping movements in the DREADD experiment. 30 min after CNO application animals did not only show a significant decline in correct placement of the paw (32.3 ± 2.5% of all analyzed grasps 30 min after i.p. CNO injection versus 54.9 ± 1.6% at baseline recordings 7 weeks post stroke, two-way ANOVA repeated measures, followed by Bonferroni post hoc test, Fig. 12A), grasping movements were also significant for the same stereotypic error patterns - too long grasps, partial slips with precent weight reinforcement of the forelimb on the wrist as well as incomplete encompassing the rung with all 4 digits (Fig. 12C-E) - which had been already detected the day after stroke and which had been classified as part of a typical graspincal failure pattern due to the injured forelimb cortex (Fig.7). Notably, the relative increase of these three grasping errors was in the same range comparing 1d post stroke to baseline with 30min after CNO application to pre-CNO baseline recordings (relative increase of 'too long grasps' 1d after stroke 9.9 ± 5.8% compared to baseline versus 9.0 ± 3.2% increase 30min after CNO compared to pre-CNO baseline; relative increase of partial slips 9.7 ± 1.2% 1d after stroke versus 9.8 ± 2.2% increase 30 min after CNO; relative increase of an aberrant closure of the paw around the rung 3.8 ± 1.2% for 1d after stroke versus 5.0 ± 1.8% increase 30 min after CNO). These results do not only indicate that the per-infarct area plays a crucial role for the recovery of skilled function after a small cortical stroke, they also emphasize that the destroyed forelimb area is completely restored by the reorganization of a new center for forelimb function in the penumbra, respectively in

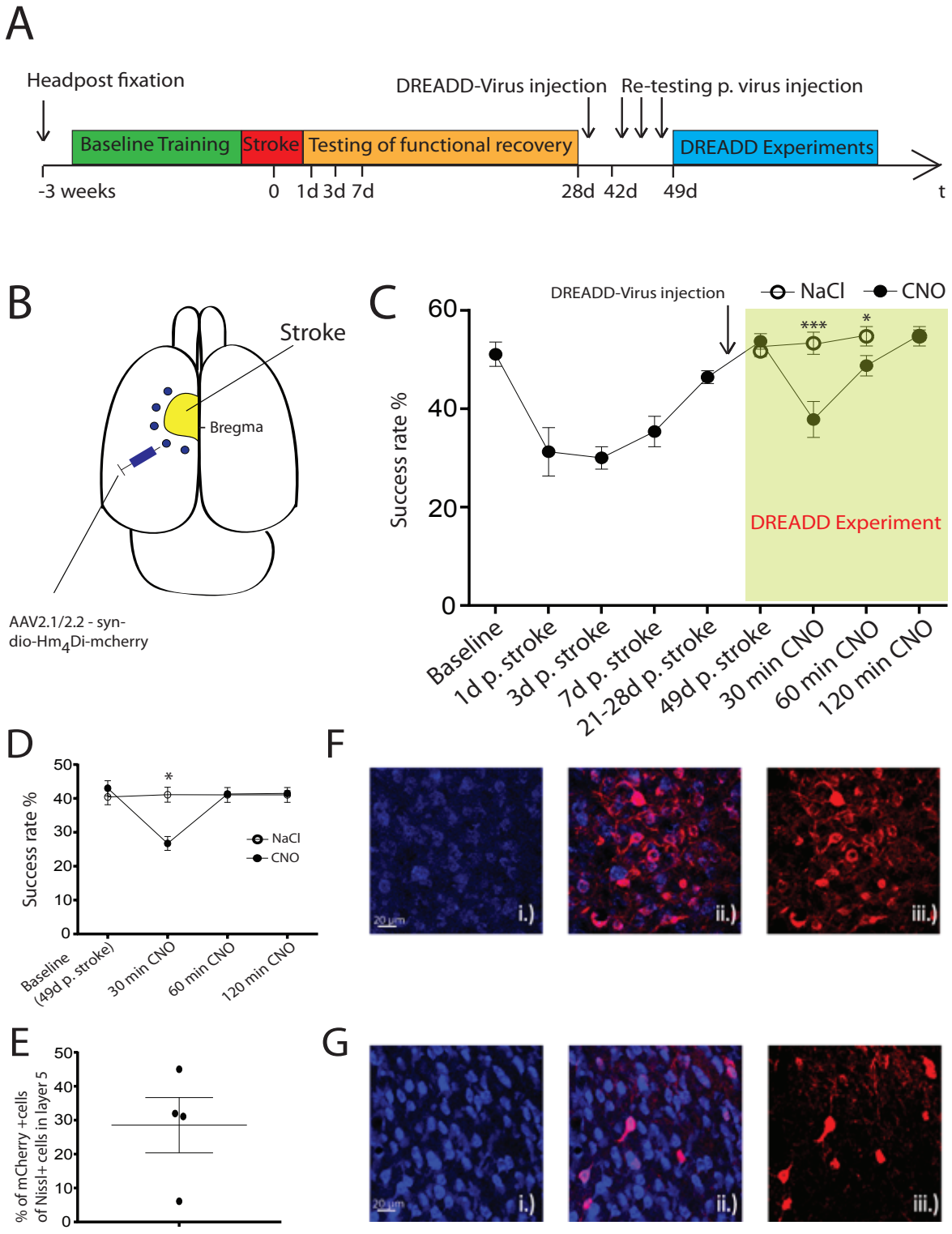

Fig. 10: The peri-infarct cortex is crucial for the restoration of skilled forelimb function after a small stroke targeting the forelimb motor cortex. (A) Scheme for the experimental time schedule. (B) Illustration showing the injection of the AAV2.1/2.2-hSyn-dio-hM4D(G_i)mCherry vector for the expression of the G_i-protein-coupled DREADD receptor hM4Di at 5 injection sites in the peri-infarct area (at 0.5 mm distance to the borders of the stroke scar) 4 weeks after stroke. (C, D) Motor performance of animals significantly declined twice within the experimental period, first after stroke induction and second, after the temporal restricted inhibition of neurons in the peri-infarct cortex 30 min after CNO application. This decrease in success rates was measured in both tests for the assessment of skilled forelimb function (the conditioned running wheel task (C) and the horizontal ladder walk test (D)). Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-way ANOVA repeated measures, followed by Bonferroni post hoc test, asterisks indicate significance: $^{**}P < 0.01$, $^{***}P < 0.001$. (E) 28.55 ± 8.13% of Nissl-positive neurons in layer five of the peri-infarct cortex were also positive for mCherry. (F, G) Representative pictures verifying the hM4Di expression (iii) in Nissl-positive layer five neurons (i) in the peri-infarct area, between 2.58 anterior (F) and -0.82 (G) posterior to bregma (ii represents the merged image of i and iii). Scale bar= 20μm.

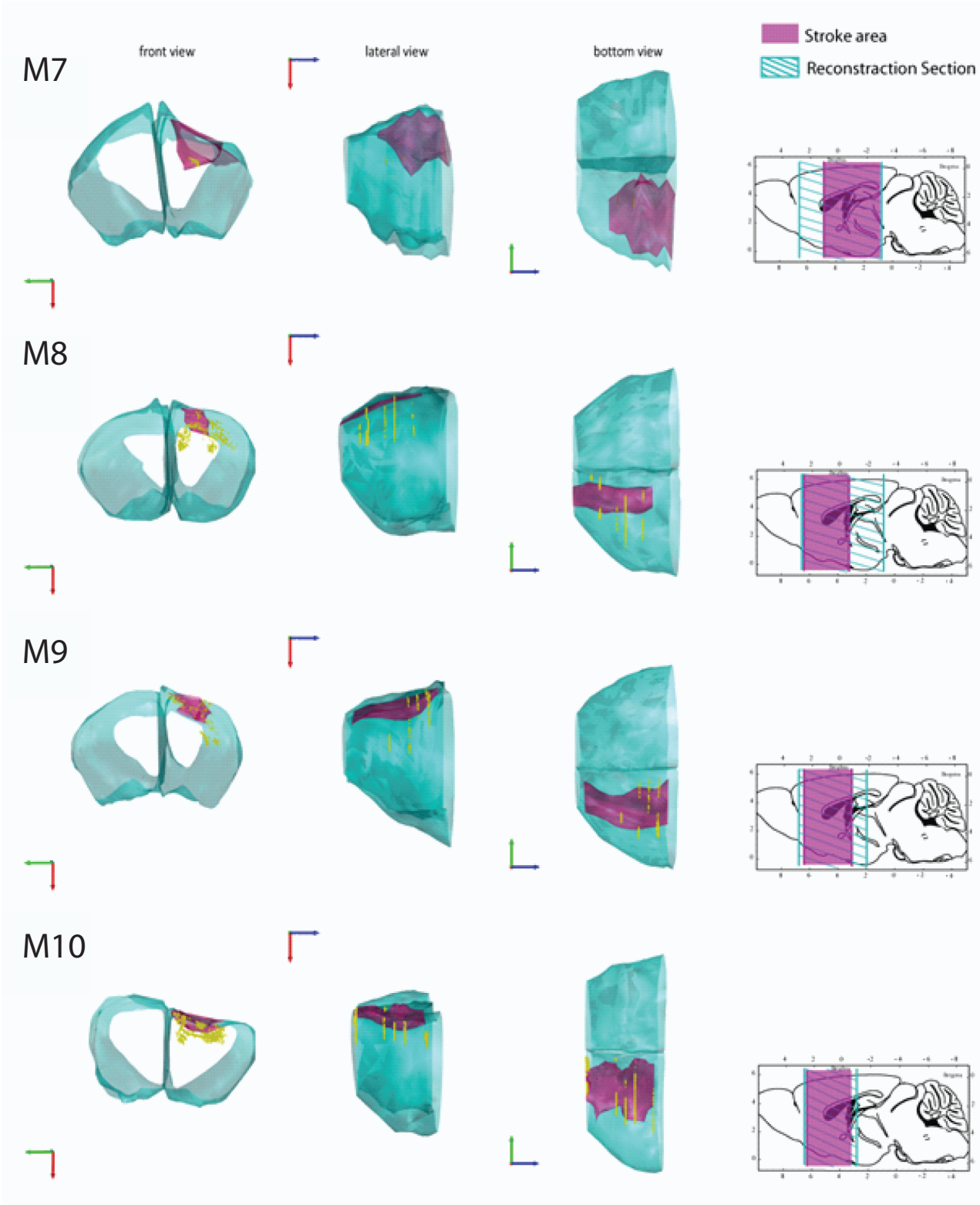

Fig. 11: Verification of neurons expressing the hM4Di DREADD receptor in the peri-infarct cortex relative to stroke lesion size using 3D neurolucida reconstruction. Two-dimensional representations of 3D Neurolucida reconstructions revealed the distribution of mCherry positive cells (yellow) in layer five of the peri-infarct area. Illustrations show the reconstructed brains of animals enrolled in the DREADD experiment (Fig. 10). In all animals the stroke was localized within the forelimb motor cortex and the mcherry positive cells were centered in the peri-infarct area. Stroke size varied from 2.68 - 1.10 anterior to -0.58-(-2.92) posterior relative to bregma. Neurolucida reconstructions were adapted from C. v. A.'s master thesis.

previous parts of the hindlimb area. Silencing this new center for forelimb function is followed by the same typical features of grasping failure as evaluated after destroying the original forelimb motor cortex area by the stroke.

3.4 Discussion

Mechanisms behind spontaneous recovery after a small stroke in the forelimb motor cortex

Our aim was to establish a small stroke through a cranial window which selectively destroys the forelimb area of the motor cortex and enables us to study reorganization of the peri-infarct tissue in ensemble with the restoration of skilled forelimb function over time. Our results indicate that the photothrombotic stroke we introduced is sufficient in size and location to specifically destroy fine motor forelimb function: Light-based mapping of the sensorimotor cortex revealed no movement responses upon stimulation of the area previously identified as the forelimb motor cortex at the 'baseline' mapping, 2 days after stroke onset (Fig. 8D) while the hindlimb motor map was preserved (Fig. 8E). Forelimb grasping function declined between 40-50% in both grasping tasks within the first week after stroke (horizontal ladder walking test, conditioned running wheel task, Fig. 5D, E) compared to the pre-stroke healthy situation. Animals then fully recovered considering their level of performance 28 days after stroke without any further intervention such as rehabilitative training, enriched rehabilitative housing or neuro-protective or growth-promoting pharmacological intervention.
Such functional recovery rates have been observed by others (Bouet et al., 2007; Alaverdashvili et al., 2008; Liu et al., 2009). This high degree of spontaneous recovery may be explained by (1) growth promoting molecular events leading to neuronal rewiring in the perinfarct cortex, (2) recruitment of subcortical motor systems and (3) by the fact that the success scores obtained in the behavioral tasks may in part not reflect true recovery but compensational events.
(1) Several studies have described that stroke triggers a specific regenerative molecular program in peri-infarct neurons (Li et al., 2010) which leads to increased brain plasticity maximally enhanced 1 week after insult which plateaus at 3 weeks (Krakauer et al., 2012). Already existent connections are recruited on an activity- and competition based-manner, new connections are formed within the peri-infarct cortex, including projections to the contralesional cortex through reduced callosal inhibition (Weiller et al., 1993, Seitz et al., 1998; Cramer, 2008; Li et al., 2011). At the cellular level, dendritic spine morphogenesis occurs in parallel with expansions in the cortical maps during the first 4 weeks after stroke (Brown et al., 2009; Mostany et al., 2010). As dendritic spines are the receiving structures for neuronal input onto pyramidal neurons (Krakauer et al., 2012), this modification of hardware structure likely plays a crucial role for providing the textural basis for the restoration of activity in the peri-infarct cortex. In addition, activity- dependent rewiring requires the alteration of electrical excitability of neurons participating in the reorganization process. Recent studies have demonstrated changes of excitatory and inhibitory receptors in the peri-infarct cortex: Low levels of AMPA receptor blockade in the first 2 weeks after stroke, which would not affect motor function in healthy mice, lead to a transient decrease in recovery (Clarkson et al., 2011). In contrast, stimulating AMPA receptor activity in the first weeks after stroke enhanced motor function. The underlying mechanism responsible for this improvement in motor recovery was revelead to occur via induction of the brain-derived neurotrophic factor (BDNF) through AMPA receptor dependent signaling.
(2)Although animals showed a significant loss of grasping ability during the first week after stroke after the center of the forelimb motor cortex had been destroyed, still stepping on the running wheel and even perfect grasps on the horizontal ladder were observable. Thus, these results indicate that other subcortical centers for motor action took over immediately or they

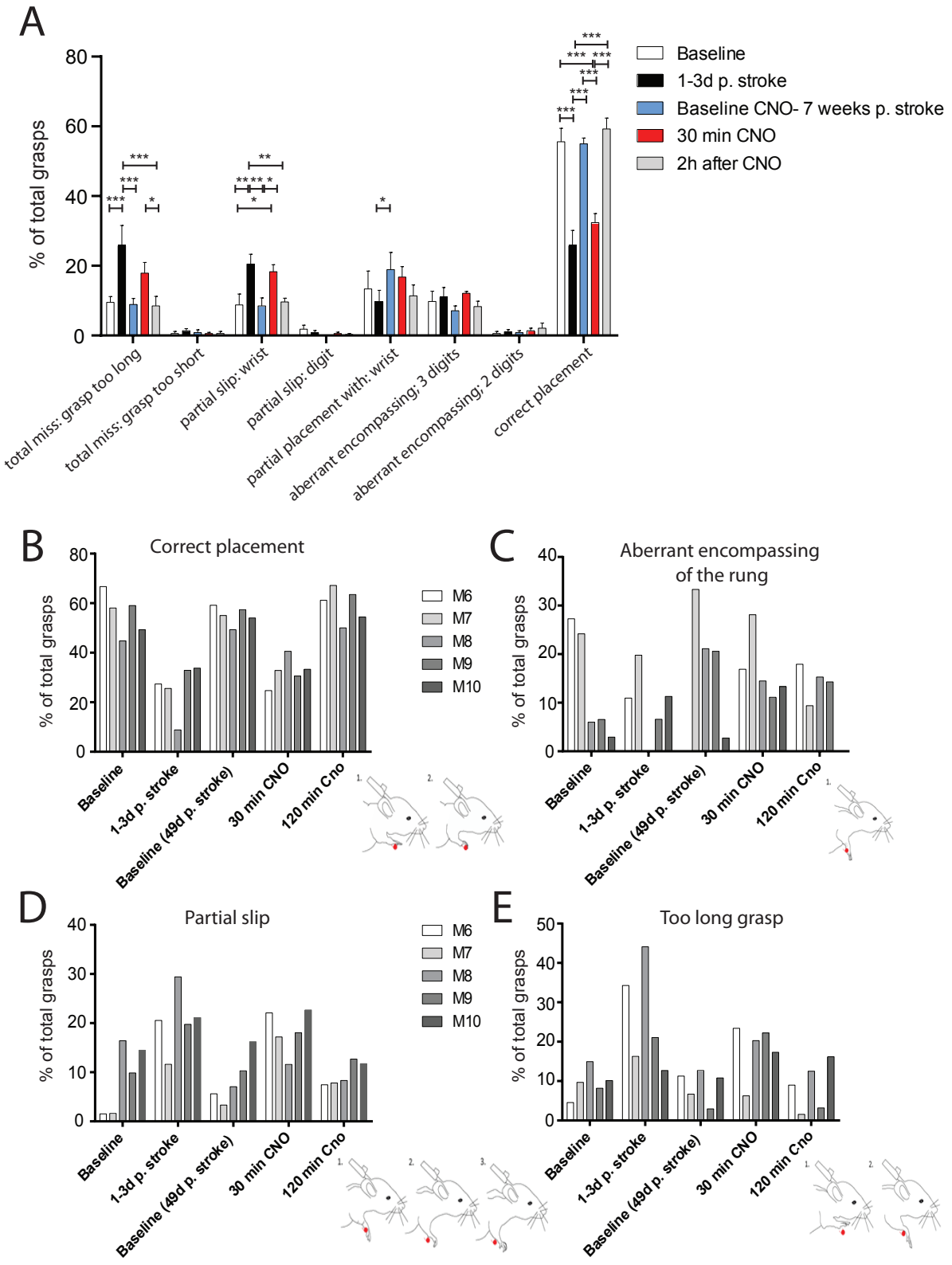

Fig. 12: Inhibition of neurons in the peri-infarct cortex resulted in the re-emergence of the same two error patterns for grasping failure as observed in the acute phase following stroke induction. (A) A score for detailed analysis of grasping kinematics revealed two significantly emerging error features (too long grasps and partial slips after targeting the rung with the wrist joint) at 1-3 days after stroke and after the pharmacogenetic inhibition of neurons in the peri-infarct area 30 min after i.p. CNO application. Both failure patterns were significantly less frequent in healthy animals at baseline recordings, in the recovered situation (4 weeks after stroke) and 2h after CNO application when the CNO concentration was scoured in the CNS). Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-way ANOVA repeated measures, followed by Bonferroni post hoc test, asterisks indicate significance: $^{**}P < 0.01$, $^{***}P < 0.001$. (B-D) Four specific grasping patterns are depicted for each animal at the five time conditions within the experimental schedule (baseline, 1-3d after stroke, baseline for the DREADD experiment (49 d after stroke), 30 min and 2h after CNO application). Percentages are given relative to the total amount of grasps analyzed for each condition. Illustrations of the grasping patterns are adapted from C. v. A.' s master thesis.

are permanently there just masked by the fine-tuned planning and controlling of movements by the motor cortex. Some researchers have claimed that the corticospinal tract might not be the major contributor to motor control in mice (Watson and Harrison, 2012) and cortical damage may be more devastating in humans than in rodent, due to the higher capability to recruit the rubrospinal and the medial system (resticulospinal and vestibulo spinal), which are larger in size and function in rodents (Bachmann et al., 2014). The corticospinal and the rubrospinal tract show many similarities, such as the somatotopical organization, with a greater representation in distal than proximal limbs, as well as similar error patterns in grasping tasks once either one or the other of the tract is impaired (Schwab and Rainteau 2001). Hence, the rubrospinal tract, originating from the red nucleus, might also contribute to distal limb control in our mice after stroke onset. Moreover, central pattern generators or propriospinal pathways as local intraspinal pathways react and adapt to optimize impaired motor control after stroke (Barrière et. al. 2008).
(3) Success scores of the grasping performance in both tasks indicated an almost full recovery of motor impairment 28 days post stroke surgery. However, the scores we used (adapted for mice from Maier et al., 2008, please refer to section 'Material and Methods' above) took only into account the targeting of the rung as a preferred-end-point model (Graziano, 2006) and did not specifically analyze grasping tajectories reflecting the sequence of action during a grasp. For these reasons the determination of the extent to which improved motor performance indicates true recovery is challenging and functional recovery may rather reflect a combination of both, true restoration of function and the activation of different subsets of behavioral compensations (Moon et al., 2009; Murphy and Corbett, 2009).

A forelimb stroke induces hyperexcitability and expansion of the hindlimb motor cortex

We used light-based mapping to study cortical reorganization in the peri-infarct cortex after a small stroke in the forelimb motor cortex over weeks. While we not only detected a map ship towards and organization of a new center for forelimb function in former parts of the hindlimb area, we also saw an expansion of the hindlimb area accompanied by increased hindlimb movement responses upon stimulation (Fig. 8D, E). Somatic map shifts are a common phenomenon after stroke described in clinical as well as experimental studies (Brion et al., 1989; Chollet et al. 1991; Ward, 2004). A study by Neumann-Haeflin et al., 1998 gave a first hind that cortical excitability might be a result of the down-regulation of the $\alpha 1$ γ-amino butyric acid receptor subunit and a decrease in γ-amino butyric acidergic inhibition. However, it remained unclear, whether map shifts introduce replacement of the former functional representation of the area the map is shifted to or whether an area maintains its primary role in addition to assuming control for functions which have been represented by the destroyed areas before. Starkey et al., 2012 found in a retrograde double tracing study that the majority of reorganized hindlimb cells which 'take over' forelimb function after a forelimb motor cortex stroke mainly connected to the cervical spinal cord. Hence, these hindlimb cells lost their original connections and instead became solely connected to forelimb output neurons in the cervical spinal cord. In contrast, Harrison et al., 2013 used longitudinal light-based motor mapping to study reorganization after a small sensory stroke, which caused a new sensory map shift to form in the motor cortex, that maintained its center position despite becoming more dispersed. Our data are in accordance to this: A new forelimb motor center was organized in a cortex area which originally represented hindlimb function. Hindlimb function of this area was preserved but was even re-organized in such a manner, that the area expanded and the threshold for stimulus evoked- motor responses of hindlimb neurons became much lower. This change of excitability may be due to modifications of inhibitory receptor density or of interneuron populations and their activity as described

(Zeiler et al., 2013).

A new center of forelimb function is organized in the peri-infarct cortex in accordance with the re-establishment of motor engrams for skilled forelimb function 4 weeks after stroke.

For many years the primary motor cortex has been viewed as a static map which can be further divided into different sub-areas for the movement control of distinct body parts, joints or even muscles while the body map is influenced by many pre-motor areas which authorize a range of higher-order functions. Based on recent findings Graziano, 2006 and others hypothesized a more complex model of the motor cortex better reflecting the highly plastic and adaptive requirements of circuit connectivity and function within the motor cortex. Graziano, 2006 claimed three major interacting levels aiming at optimal motor control to perform a motor task:
(1) The motor cortex may be divisible into zones that emphasize different behaviorally relevant categories of movement instead of a somatotopic map which separates movements into constituent muscles and joints.
(2) A motor neuron in the cortex may not connect to a single muscle in the periphery but rather excites interneuron pools in the spinal cord resulting in the activation of muscle synergy groups. A motor neuron cannot be simply classified for a specific direction tuning component, speed, force angle and muscle activity - although in specific contexts correlations have been described (Evarts, 1968; Cheney et al., 1985; Georgopoulos et al., 1992; Holdefer and Miller, 2002; Kakei et al., 1999) - instead, single neurons in the motor cortex may be tuned in an idiosyncratic fashion to complex, behaviorally useful patterns of motor output which reflect common actions. Thus, motor neurons are direction tuned within a specific meaningful task which can rapidly change as the task changes - neurons are locally tuned, but globally not. 'Within this optimal control theory, there is no single preferred parameter for direction, end posture or joint speed. Instead, the parameters being controlled depend on the task being performed.' (Graziano, 2006).
(3)The spinal cord controls movements at a level of complexity which cannot be summarized on the level of muscle maps or joints proposed for fine-tuning by the primary motor cortex. Thus, the connectivity between the motor cortex and muscles is not fixed but fluid, changing constantly on the basis of feedback from the periphery.
This modern view of motor cortex - spinal cord interaction and computation may allow us to explain the high capacity of the motor system to learn new tasks and to be adaptive after damage to a part of the circuitry.
In this study we used the horizontal ladder walking test and a modified variant that even required more precise targeting and coordination of muscle strength, the conditioned running wheel task, to assess skilled forelimb function. All animals were pre-trained in those tasks to achieve at least 60% success rates before a photothrombotic stroke was introduced. Motor learning of a task usually consists of two components, a slow and a fast process to enhance motor performance. The slow one occurs during the first few trials of the training session whereas the fast process develops over time across sessions (Tucci et al., 2007). While mice were training on the wheel, their grasping increased in accuracy accompanied by correct dedication of their body weight, leading to improved balance control and coordination of limb strength over the course of time. Such improvements suggest that grasping and stepping performance employs complex brain connectivity (Tuccis et al., 2007). In addition, training of a motor task induces not only changes in motor map organization but also establishes specific motor engrams which reflect the acquired skills (Monfils et al., 2005). Thus, training on the wheel or horizontal ladder may emphasize connections which lead to meaningful action patterns, such as adjustments of

the paw placement according to the rung sequences.
When evaluating post stroke performance of mice we found two stereotypical grasps, which were repetitively detected, in particular within the first week after stroke: over-extensive grasps (missing the rung) and partial slips with the wrist. Hence, the stroke destroyed the motor map organization resulting in a loss of the cortical network structure responsible of correct paw placement in the preferred-end-posture model: Due to a defective optimal control strategy accurate paw placement was lost and grasps became to long.
In addition, the lesion in the motor cortex of the forelimb area damaged layer 5 output-neurons, which targeted axons in the subcortical area, involved in precise motor movements. As the corticospinal pathway originating from layer 5 neurons, regulates sequential and synergetic movements of each muscle to control fine motor movement, especially the target reaching posture (Ueno and Yamashita, 2011), the stepping on the irregular rung pattern required sequences of skilled movements: First the targeting trajectory of the paw for the appropriate rung followed by the accurate placing and finally, the closure of the paw around the rung with consecutive contraction of flexion muscles within the elbow joints for the movement of the rest of the body (Metz and Whishaw, 2002). In our study mice were able to place the paw on the rung but were unable to close the paw and to stabilize body posture before moving towards the next rung. Instead we detected extensive usage of wrist/heel retaining movements with coarse motor actions leading to subsequent slips. These observations indicate a failure of the target end posture, but may also be explained as a result of impaired sensory perception, of inter-limb coordination and the inability to perform balanced weight supported stepping movements after damage in the sensorimotor area.

28 days after stroke animals depicted full recovery of success rates in both grasping tasks and the two stereotypical error - too long grasping and slipping after targeting the rung with the wrist - had disappeared (Fig. 5). However, when we used a pharmacogenetic approach to temporarily and selectively inhibit neurons in the per-infarct regions (Fig. 10), both types of grasping errors re-emerged. This result not only confirms the re-organization of the center for forelimb motor control in the peri-infart area, something, which we have already seen during light-based mapping over the course of 4 weeks after stroke surgery (Fig. 8), but also suggests the re-establishment of specific motor engrams for skilled grasping function, meaning the rewiring of sets of neurons tunable to form networks for precise sub-types for forelimb function within the newly re-organized forelimb area. Neurons in the peri-infarct hindlimb cortex were able to take-over meaningful forelimb function maintaining their hindlimb function. Thus, our study provides new evidence for a sensorimotor cortex whose computational processing is very adaptive and tunable, and which, instead of categorizing neurons in fixed specializations for distinct movements, emphasizes behaviorally relevant neuronal circuit formation.

Conclusion and Outlook

In summary, this study indicates the crucial importance of the peri-infarct area for the restoration of skilled forelimb function after a small stroke destroying the forelimb motor cortex. Within 4 weeks after stroke the center of forelimb function is re-organized within a part of the former hindlimb motor cortex accompanied by a full recovery of forelimb function. Temporarily inhibiting neurons in the peri-infarct region lead to a decline of regained fine motor skills and a re-emergence of stereotypical error patterns for grasping as detected immediately after stroke. These results suggest a full establishment of motor engrams for grasping function in the former hindlimb cortex. However, there are limitations to this study: Light-based mapping of the sensorimotor cortex, although an improvement compared to the invasive intracranial microstimulation, may always provide only rough map shifts while an approach which studies neuronal networks on the level of single cell resolution opens up a broad range of possibilities to

directly study formation, loss and re-emergence of specific motor engrams - even in dependence of external rehabilitative interventions - before and after stroke.

3.5 References and Notes

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Chapter 4

Asynchronous therapy restores motor control by rewiring of the rat corticospinal tract after stroke

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A.S.W. designed, conducted, analyzed and interpreted all the experiments. Concept, manuscript and figures by A.S.W. with help of M.E.S.

Collaborators: W.O. provided matlab scripts for ICMS data analysis. J.C.R., H.Z. and B.O. developed computer and machine learning algorithm tools for data analysis. J.L.C. and K.K. provided virus vectors. A.S. performed MRI imaging. M.G. implanted the osmotic pumps. O. W. developed the protocol for histological analysis. B.O. and F.H. helped preparing figures and writing the manuscript.

One sentence summary

Based on a distinct rehabilitation paradigm after stroke in adult rats we stimulate the formation of new neuronal circuits that restore motor function and prove their functional relevance with two diverse pharmacogenetic approaches for reversible blockade of the fibers from the forebrain cortex to the spinal cord.

Abstract

The brain exhibits limited capacity for spontaneous restoration of lost motor functions after stroke. Rehabilitation is the prevailing clinical approach to augment functional recovery, but the scientific basis is poorly understood. Here we show almost full recovery of skilled forelimb functions in rats with large strokes when a growth promoting immunotherapy against a neurite growth inhibitory protein was applied to boost the sprouting of new fibers, before stabilizing the newly formed circuits by intensive training. In contrast, early high intensity training during the growth phase destroyed the effect and led to aberrant fiber patterns. Pharmacogenetic experiments identified a subset of corticospinal fibers originating in the intact half of the forebrain, side-switching in the spinal cord to newly innervate the impaired limb and restore skilled motor function.

Stroke is a major cause of severe disability in the elderly population, and recovery after large strokes is limited (Murphy and Corbett, 2009; Dimyan et al., 2011). Current strategies to improve long-term outcome in humans include mostly rehabilitative training and in experimental models also electrical stimulation and pharmacological interventions (Langhorne et al., 2011). However, all of these treatment options have had only limited success so far (Carmichael, 2006; Zeiler and Krakauer, 2013). Here we show that rehabilitative training, if preceded by a nerve growth-promoting antibody therapy almost completely restored skilled forelimb functions after cortical strokes in adult rats. Sequential application of the treatments was essential: when growth promotion by blockade of the neurite growth inhibitory protein Nogo-A was simultaneously applied with intensive forced-use training of the forelimb during the first 2 weeks after the stroke, functional outcome was poorer compared to training, immunotherapy alone or no treatment at all (Lindau et al., 2014). Anatomically, Nogo-A neutralization promoted growth of corticospinal fibers from the intact forebrain motor cortex across the midline of the cervical spinal cord. In rats with simultaneous antibody treatment and training, fiber branching was exuberant with anatomically aberrant terminations. In contrast, in animals trained for forelimb function subsequent to antibody treatment, axonal fibers originally terminating in the intact spinal hemicord crossed the mid-line and innervated the ventral motor regions of the spinal hemicord that had lost its input from the motor cortex. To prove the functional relevance of these newly grown, 'side-switched' descending corticospinal tract fibers, we selectively and temporarily blocked these fibers by two different pharmacogenetic techniques: both suppressed the restored forelimb function. Our results demonstrate that a sequential strategy of first promoting fiber growth to enhance the low endogenous plastic potential of the brain and spinal cord followed by rehabilitative training-induced selection and stabilization of functionally meaningful connections can lead to much higher levels of functional restoration after large brain lesions than currently obtained in conventional rehabilitation medicine.

4.1 Success of rehabilitation depends on timing

We compared four different therapy and rehabilitation schedules for promoting functional recovery of fine motor skills of forelimbs in a thrombotic stroke model in rats. Using the well-established technique of photothrombosis (Lindau et al., 2014), we induced blood vessel blocks by multiple microthrombi which destroyed $> 90\%$ of the sensory-motor cortex of adult rats. Rats were then treated by intrathecal anti-Nogo A or control antibody for 2 weeks (Lindau et al., 2014; Oertle et al., 2003). In addition, we trained rats intensely in skilled forelimb reaching (100 reaches per day) either simultaneously with antibody application ('parallel' groups) or during the two weeks after antibody treatment ('sequential' groups) (Fig. 1A). To avoid a training effect of testing itself we did not re-assess the sequential groups ($'anti-Nogo-A/sequential'$ and $'controlsequential'$) during the first two weeks post lesion. When the growth enhancing anti-Nogo-A treatment was followed by the rehabilitative training ($'anti-Nogo-A/sequential'$ group), animals improved their performance from day 16 post-stroke onwards, and their skilled reaching abilities almost completely recovered(Fig.1A, B; reaching $86.3 \pm 2.0\%$ of pre-stroke level; significantly better than all other groups; $p < 0.001$, two-way repeated measures ANOVA with posthoc Bonferroni). This group also performed best in two novel tasks of skilled forelimb use tested at the end of the experiment (Montoya staircase grasping: success rate $34.1 \pm 5.1\%$; Fig. 1C; horizontal ladder crossing: success rate $65. \pm 3.7\%$; Fig. 1D). As animals had not been exposed to these tasks before, these results indicate a generalization of the recovery of forelimb function in the sequential training group that is transferable to non-trained motor skills.
In contrast to these results, rats receiving intensive forelimb training concurrently to the anti-Nogo-A antibody treatment ($'anti-Nogo-A/parallel'$ group) performed worse than all other groups in the single pellet grasping task (success rate $10.0 \pm 5.2\%$, Fig. 1A, B). In the novel

tasks tested at the end these animals showed either no significant improvement (Fig. 1C) or a tendency of decline over the course of trails ($p = 0.1$, two-way repeated measures ANOVA with posthoc Bonferroni, Fig. 1D). The control antibody treated groups reached low levels of recovery ($35 - 40\%$ success rate in pellet grasping, Fig. 1 $A - D$), with an early training effect visible in the group trained during the first two post-stroke weeks ($'control/parallel'$, Fig. 1A). Final success scores did not correlate with stroke volume as determined ex vivo either from whole-brain MR-images ($r = 0.04$, Spearmann correlation; Fig. 1F) or histological Nissl stains (Fig. 2), suggesting no specific neuroprotective effect by any of the four therapeutic schedules. We conclude that applying a nerve fiber growth-promoting cellular therapy and rehabilitative physical training in sequence delivers greater functional recovery than when the same protocols are applied concurrently.

4.2 Rehabilitative schedules induce distinct neuronal fiber patterns

We investigated the neuroanatomical correlates of functional recovery by labeling the intact, contralesional corticospinal tract, which normally innervates the spinal cord half opposite to the one which has lost its cortical input, with only few fibers crossing the spinal cord midline. Each of the four experimental groups presented a unique rehabilitation-induced pattern of fiber sprouting in the cervical spinal cord (Fig. 3C). We counted labeled fibers originating in the cortex of the intact side opposite to the stroke and crossing the midline of the spinal cord (Fig. 3A). We also quantified their elongation and branching within the grey matter of cervical spinal cord, i.e. the cord region containing the motor control circuits of the forelimb and paw (Fig. 3A,B). The greatest number of midline-crossing fibers was seen in the $'anti - Nogo - A/sequential'$ treatment group, which also had the best functional outcome. In contrast, the $'anti - Nogo - A/parallel'$ group, showed extensive branching of the midline crossing corticospinal fibers (Fig 3B, $p < 0.05$, two-way repeated measures ANOVA with posthoc Bonferroni).

A quantitative analysis of the distribution and density of ipsilaterally projecting corticospinal fibers using pattern recognition algorithms to analyze both single corticospinal fibers and related fiber growth parameters (see Methods) confirmed overshooting fiber growth and aberrant termination patterns in the $'anti - Nogo - A/parallel'$ group. In the $'anti - Nogo - A/sequential'$ group midline-crossing sprouting corticospinal fibers displayed a radial organization with few branches and a preference for the premotor and motor spinal cord (laminae $6-9$, Fig. $3D-G$).In contrast, fibers in the $'anti - Nogo - A/parallel'$ group appeared less organized with more than double the number of branches and a different laminar distribution including the dorsal, predominantly sensory laminae $1-5$. We also assessed the connectivity of the ipsilaterally projecting corticospinal fibers by quantifying the density of axonal boutons recognized morphologically in the premotor interneuron lamina 7: A significantly higher bouton density was detected in the $'anti - Nogo - A/parallel'$ group compared to the $'anti - Nogo - A/sequential'$ group (Fig. 3H; $p < 0.05$, Student‘s t-test, two-tailed, unpaired). The $'anti - Nogo - A/parallel'$ group showed a greater tendency of axons to grow beyond the grey/white matter boundary, and a highly aberrant growth pattern (Fig. 3H, I). In the medio-ventral funiculus, such fibers are probably intermixed with sprouts of the small, uncrossed ipsilateral corticospinal tract.

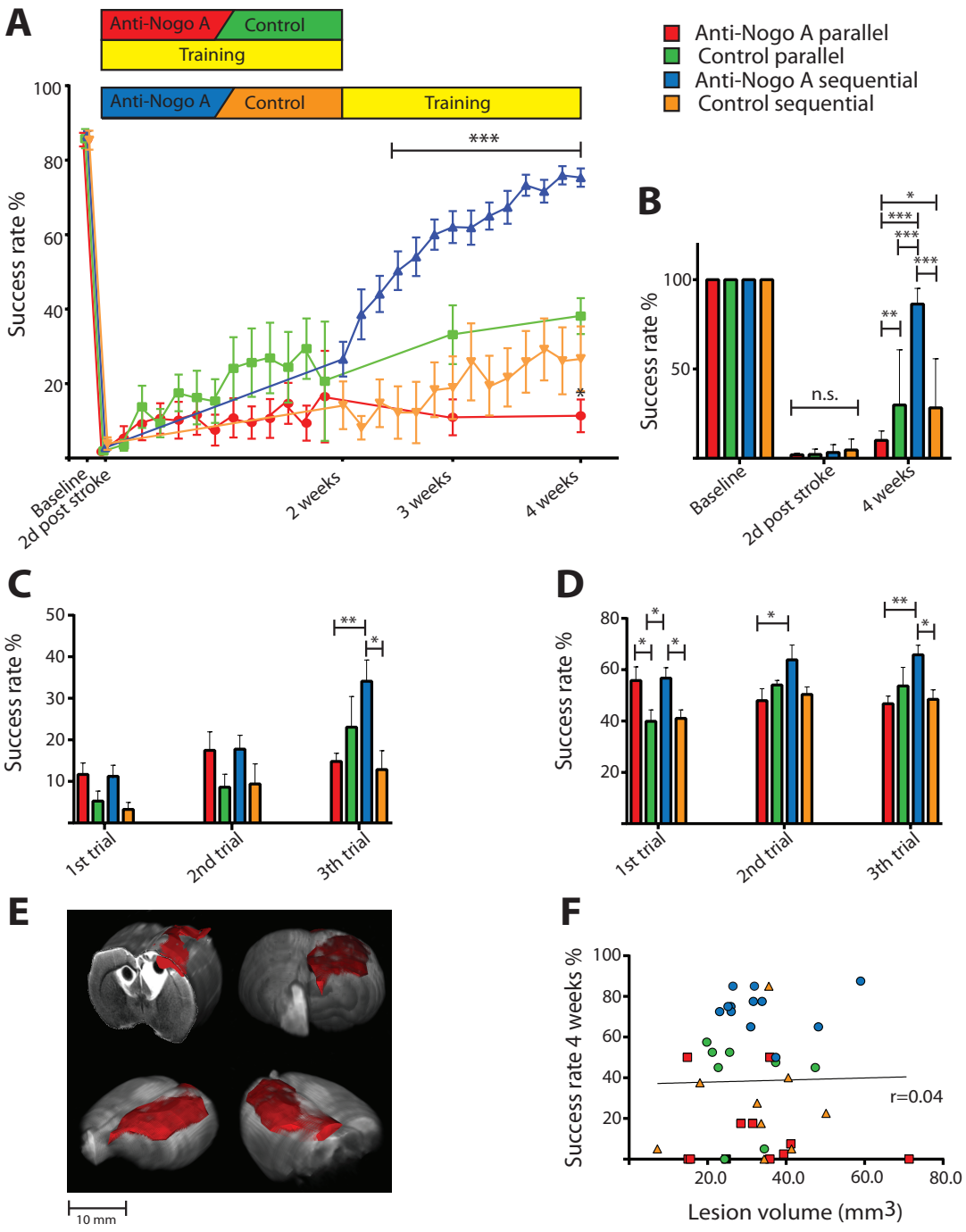

Fig. 1: Timing matters when a growth promoting anti-Nogo A immunotherapy is combined with training. (A) Success rates in the single pellet grasping task at baseline (intact, trained), 2 days after a large, unilateral photothrombotic stroke to the sensorimotor cortex of the preferred paw, and during training and re-testing sessions until 4 weeks post-insult. The $'anti-Nogo-A/sequential'$ group showed significant improvement compared to all other groups whereas the performance of the anti-Nogo-A/parallel group was significantly worse ($'anti-Nogo-A/parallel'$, n=16; $'control/parallel'$, n=8; $'anti-Nogo-A/sequential'$, n=16; $'control/sequential'$, n=9). (B) Recovery rates expressed as success rates of last testing session normalized to baseline performance (100%). (C, D) Animals in the $'anti-Nogo-A/sequential'$ group also performed significantly better in novel grasping tasks such as the Montoya staircase test (C) or the horizontal ladder crossing task (D) introduced after the completion of the rehabilitation schedules for 3 consecutive trails. Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-way ANOVA repeated measure followed by Bonferroni post hoc, asterisks indicate significances: $^{*}P < 0.05$,$^{**}P < 0.01$, $^{***}P < 0.001$. (E) Representative picture of an ex vivo MRI image stack for 3D stroke reconstruction. (F) There was no correlation between stroke volume and end point success rate in the single pellet grasping task among all rehabilitation groups ($r = 0.04$, Spearman correlation).

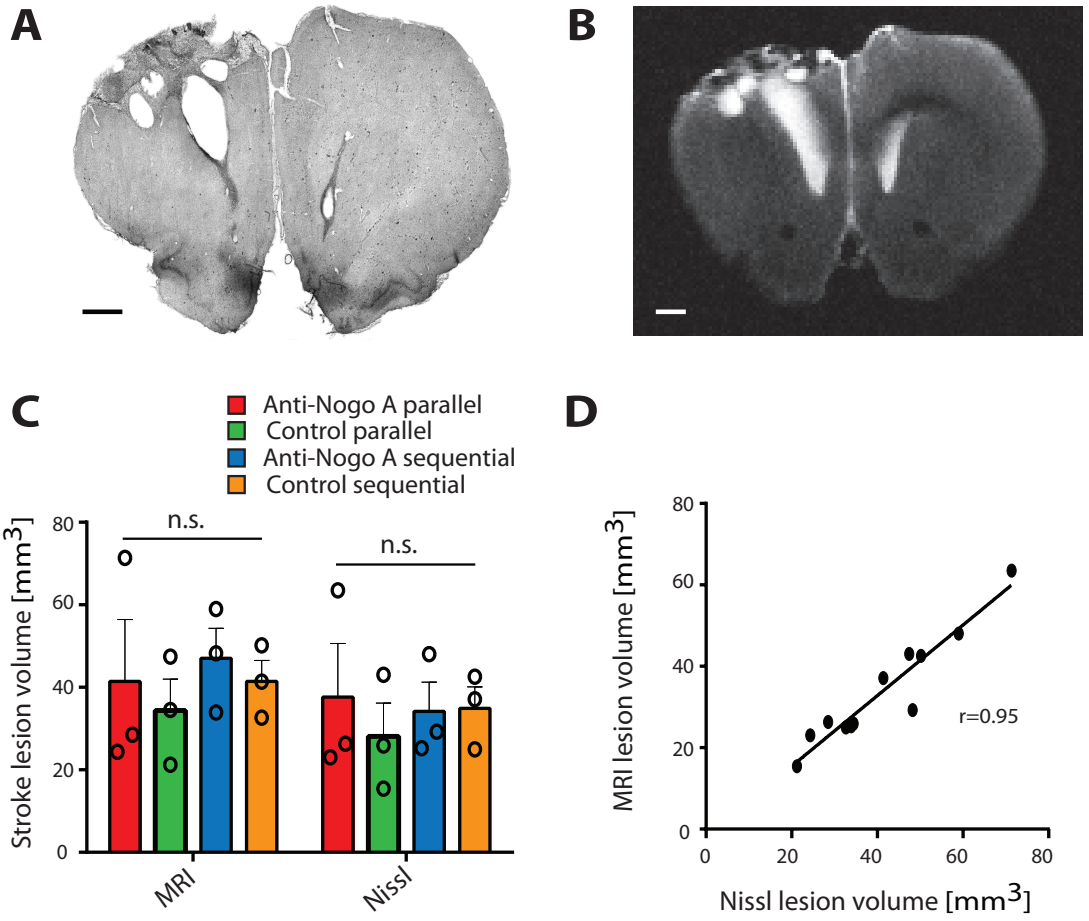

Fig. 2. Histological analysis and MRI imaging of stroke volume reveals no difference in lesion size among rehabilitation groups. (A, B) Representative coronal sections of a brain after the completion of rehabilitative treatment 8 weeks post a photothrombotic stroke for analysis of stroke volume using histological Nissl staining (A) and MRI imaging (B) (scale bar= 1000 μm). (C) Stroke lesion size did not differ among the four different rehabilitation groups using both MRI imaging and 3D Neurolucida reconstructions of coronal sections after Nissl staining (animals per rehabilitation group, n=3, r=0.95 Pearson correlation). (D) Stroke volumetry on MRI images determined by a compositional registration and warping algorithm developed to extract the shape of the lesion and to measure its size correlated with histological analysis of Nissl stained sections. Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-way ANOVA repeated measure followed by Bonferroni post hoc (C).

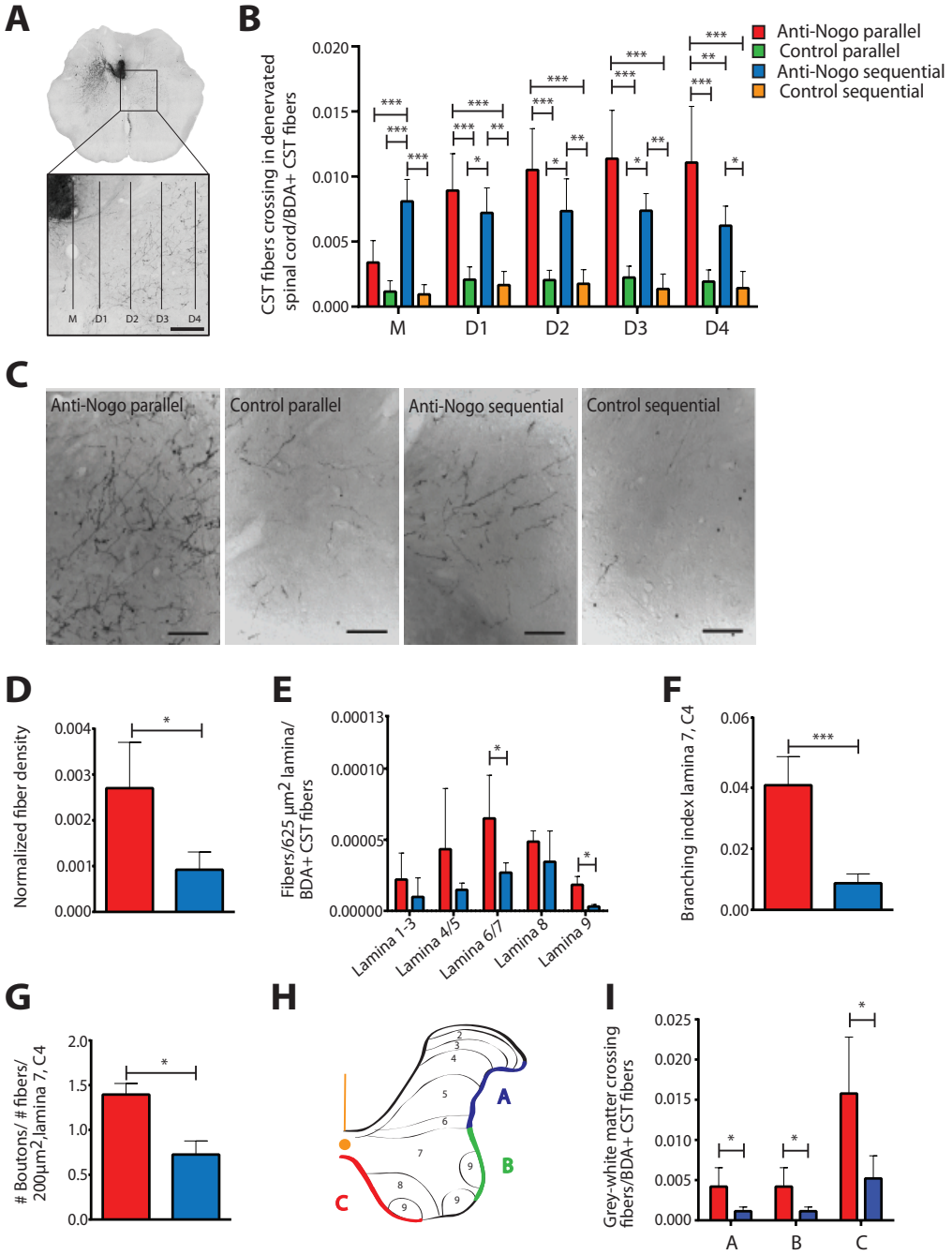

Fig. 3: Corticospinal tract sprouting depends on timing of rehabilitative training correlating with functional recovery. The four rehabilitation schedules ($'anti-Nogo-A/parallel'$, n=10; $'control/parallel'$, n=8; $'anti-Nogo-A/sequential'$, n=10; $'control/sequential'$, n=8) differently influenced the sprouting of corticospinal fibers from the intact side of the spinal cord across the spinal cord midline (M). (A) Low and high magnification micrographs of BDA-labelled corticospinal fibers in intact spinal hemicord (left) growing into the stroke-denervated hemicord (right; inset) at spinal cord level C4. D1-D4: Lines for intersection counts with corticospinal fibers (scale bar=200 μm). (B) Fibers crossing the midline (M) and branching in the grey matter at distances D1-D4 were counted and normalized to the number of BDA-positive labelled fibers in the main tract. (C) Micrographs showing different sprouting patterns of corticospinal fibers from the ipsilateral cortex in the denervated cervical spinal cord (C4) in lamina 7 in the different treatment groups (scale bar=200 μm). (D) Combining anti-Nogo-A immunotherapy with simultaneous training ($'anti-Nogo-A/parallel'$) results in a significantly higher density of ipsilateral CST fibers in the stroke-denervated cervical spinal cord than anti-Nogo-A/sequential treatment. (E) The most significant difference in fiber density between $'anti-Nogo-A/parallel'$ and $'anti-Nogo-A/sequential'$ animals was detected in lamina 6/7 and lamina 9 of the denervated cervical hemicord. Lamina 7 was also significant for increased fiber branching (F) (branching index=branches per fiber/BDA positive fibers in the intact CST) and bouton numbers (G) in the $'anti-Nogo-A/parallel'$ group. (H, I) Significantly more fibers cross the grey-white matter boundaries in the dorsolateral (A), the ventrolateral (B) and the ventro-medial funiculus (C, scheme shown in H) in the anti-Nogo-A/parallel group. Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-way ANOVA repeated measure followed by Bonferroni post hoc (b) and Student´s t-test (two-tailed, unpaired) for e-j, asterisks indicate significances: $^{*}P < 0.05, ^{**}P < 0.01$, $^{***}P < 0.001$.

4.3 Nerve cells from the intact forebrain cortex are responsible for recovery

Our results suggest that the recovery of rat forelimb function after stroke in the $'anti - Nogo - A/sequential'$ group originates from extensive and precise re-innervation of the stroke-denervated spinal hemicord by midline-crossing fibers from the intact motor cortex and corticospinal tract. We tested this hypothesis in the animals of the $'anti - Nogo - A/sequential'$ group, all of which showed excellent functional recovery, by using two different experimental approaches for inducible, selective and reversible inactivation of the ipsilaterally projecting corticospinal fibers on the long and short term, respectively. For long-term blockade we used virus to deliver a doxycyclin-inducible tetanus toxin to temporarily inactivate the synaptic release mechanism (Kinoshita et al., 2012). We injected the highly efficient retrograde gene transfer lentivector HiRet carrying enhanced tetanus neurotoxin light chain (eTeNT) with an enhanced green fluorescent protein (EGFP) downstream of a tetracycline-responsive element (TRE) into the stroke-denervated side of the cervical spinal cord at level C5-C6, and the adeno-associated serotype 2.2 (AAV2) vector carrying the reverse tetracycline transactivator (rtTAV16, Tet-on) into the contralesional, intact pre-motor and motor cortex ($n = 6$, Fig. 4A, B). Only cortical neurons with axons projecting to the stroke-denervated spinal cord would contain both transgenes and activate tetanus toxin in response to doxycycline. We applied the same procedure in 4 control animals except that these animals received injections of the HiRet lentivector coding for EGFP only.
After recovery from the surgery and the reassessment of re-gained grasping skills, doxycycline was orally administered for two weeks (Fig. 4A, C). In the experimental group grasping performance declined within a few days reaching a very low level from day 7 after doxycycline initiation ($p < 0.05$, statistical comparison TeNT versus control group, two-way repeated measures ANOVA with posthoc Bonferroni; Fig. 4C).When the drug administration was ceased, the lost function was regained within 2 weeks. All animals again showed a loss of skilled food pellet grasping movements when oral doxycycline intake was re-started for a second time over the course of another 3 weeks (Fig. 4C). No deterioration of the post-stroke recovered skilled grasping was observed in control animals (EGFP instead of eTeNT) under the same dosage of doxycycline and within the same time frame ($98.1 \pm 0.9\%$ of pre-doxycycline grasping performance). As the retrogradely transported virus was EGFP tagged, corticospinal neurons projecting to the ipsilateral cervical spinal cord segments C5-6 could be quantified. These neurons were concentrated in layer 5 of a specific region of the rostral, premotor and the primary (M1) forelimb motor cortex ($3.7mm \pm 0.2$ anterior to bregma). In the center of the labeled region, $41.4 \pm 5.6\%$ of Nissl-positive cells of layer 5 contained the transgene (Fig. 4D, E). These results demonstrate that midline-crossing corticospinal fibers from the intact hemisphere opposite to the stroke lesion anatomically and functionally switch sides, which is crucial for the recovery of skilled forelimb movements. They maintain their new function, even if they are functionally blocked for weeks. Evidently, their role cannot be compensated by other cortical or subcortical motoneuronal pathways in the injured system.

4.4 Temporally blocking rewired corticospinal fibers results in decline of regained function.

For short-term reversible inactivation of the midline-crossing corticospinal fibers we used a pharmaco-genetic approach involving virus-mediated gene transfer to express engineered Gi/o-coupled DREADD receptors (‘designer receptor exclusively activated by designer drug‘). These receptors are only activated by the otherwise pharmacologically inert synthetic ligand clozapine-

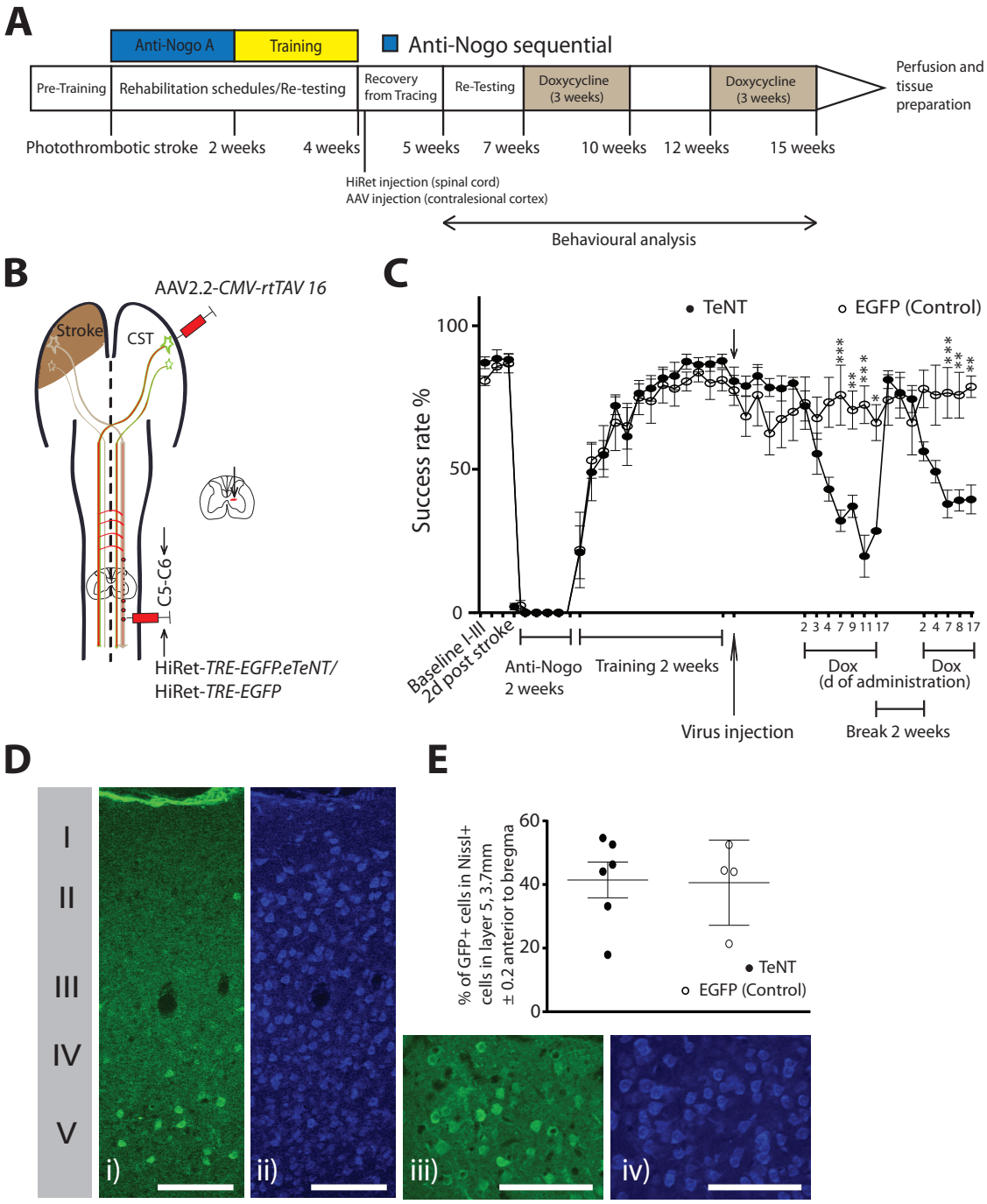

Fig. 4: Long-term reversible blockade of midline crossing corticospinal fibers abolishes the functional recovery after stroke. (A) Experimental schedule; all animals had anti-Nogo-A immunotherapy followed by intensive reaching training, leading to almost full functional restoration of skilled forelimb functions. (B) Schematic diagram of vector injections into the contralesional motor cortex (AAV2.2-*CMV-rtTAV*-16) and stroke-denervated cervical spinal cord (HiRet-*TRE-EGFP-TeNT* or HiRet-*TRE-EGFP* as control). (C) Induction of tetanus toxin by doxycycline leads to strong impairment of reach and grasping movements within 7days. The effect was fully reversible within 7-10 days of dox removal and could be reinduced by re-application of dox. TeNT animals n=6, control animals n=4. (D) Examples of GFP positive cells (i) in the rostral sensorimotor cortex, 3.7 mm anterior to bregma in comparison to Nissl positive cells (ii) (i, ii: scale bars= 100μm) and zooming in to GFP positive cells in layer 5 (iii) in relation to Nissl positive cells (iv) (iii, iv scale bars= 50mum). (E) Quantification of GFP positive cells in percentage of Nissl positive cells in layer 5 motor cortex. Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-way ANOVA repeated measure followed by Bonferroni post hoc (b) and Student´s t-test (two-tailed, unpaired) for e-j, asterisks indicate significances: $^{*}P < 0.05$,$^{**}P < 0.01$, $^{***}P < 0.001$.

N-oxide (CNO) resulting in increased intracellular Gi/o –mediated signaling which leads to membrane hyperpolarization and silencing of the infected neurons (Conklin et al., 2008; Alexander et al., 2009; Ferguson et al., 2011). We used only rats that had undergone the treatment schedule of growth promotion by Anti-Nogo-A antibodies followed by another 2 weeks of rehabilitative training. The virus injections started at the end of the training period (Fig. 5). To selectively manipulate the corticospinal fibers projecting from the intact, contralesional motor cortex to the denervated cervical spinal cord, we first injected an adeno-associated serotype 2.9 (AAV2.9) vector carrying the Cre sequence into segments C5 and C6 of the stroke-denervated cervical spinal cord, followed by injections of the Cre-dependent AAV2.1 vector carrying the Gi/o-coupled DREADD (hM4Di) receptor into the contralesional pre- and sensorimotor cortex ($n = 6$, Fig. 5B). The DREADD receptor hM4Di was tagged with mCherry, which allowed neuroanatomical confirmation that only double-infected cells expressed the hM4Di receptor (Fig. 5C): We found mCherry- positive cells concentrated in layer 5 of the same region of the contralesional premotor and motor cortex ($3.7mm \pm 0.2$ anterior to bregma) as in the tetanus toxin experiment. $31.6 \pm 2.4\%$ of Nissl-positive layer 5 cells in this region were mCherry-positive (Fig. 6A, B), with very limited numbers of mCherry-positive cells outside of this area. In control animals that received injection of the Cre-dependent AAV2.1-hM4Di vector in the contralesional cortex without AAV-Cre-virus injection in the spinal cord, only $4.3 \pm 0.5\%$ of Nissl-positive cells in layer 5 were positive for mCherry indicating low background noise ($n = 4$, Fig. 6A).
Three weeks after virus injection both hM4Di/+Cre and control hM4Di/-Cre animals performed at $> 80\%$ of success rate in single pellet grasping (Fig.6C). Animals were then injected intraperitoneally with the channel activating drug CNO. Control animals showed no change in reaching and grasping abilities over the 50 minutes of observation time. However, animals of the hM4Di/+Cre group lost their grasping abilities over 10-30 minutes, with performance declining to $38.9 \pm 6.0\%$ success rate, significantly lower compared to the control group (Fig. 6C; $p < 0.001$, two-way repeated measures ANOVA with posthoc Bonferroni). The defective movements were characterized by a specific failure to target the paw to the pellet and to close the paw around the pellet (Fig. 6D), but with little modifications in overall grasping trajectories (Fig. 7). The abatement of distal motor functions was confirmed by performing discriminative classification based on a non-parametric representation (Ommer et al., 2009) of paw posture and its change over time ($p = 0.02$, grasps at 'baseline' versus 'after 30 min CNO', K-S-Test). This mainly distal impairment may also be due to the fact that only the cervical segments C5 and C6 were injected with the Cre-virus. These functional defects were fully reversible, with performance returning to pre-injection levels about $40 - 50$ min after the CNO injection (Fig. 6C, D).

4.5 Pharmacogenetic inhibition of regained EMG activity

We confirmed the CNO-specific blockade of neuronal firing of ipsilaterally projecting, in part midline-crossing corticospinal fibers of the intact, contralesional motor cortex by electrophysiology using intracortical microstimulation (ICMS) at the end of the behavioural testing: We used a $5x12$ point stimulation grid (positioned at +3 to -3 mm anterio-posterior and 1 to 3.5 mm medio-lateral relative to bregma, Fig. 8A) and electromyogram (EMG) recordings of wrist, elbow and shoulder muscles of the impaired paw as readouts (Fig. 8B). In all animals each cortical position within the exploration grid was stimulated twice, first as baseline stimulation, then again 30 min after CNO injection. In all hM4Di/-Cre control animals the EMG responses at baseline and 30 min post CNO injection were not significantly different for the 60 stimulation points (for wrist 95% confidence interval between 'baseline' and '30 min CNO' was for animal 1

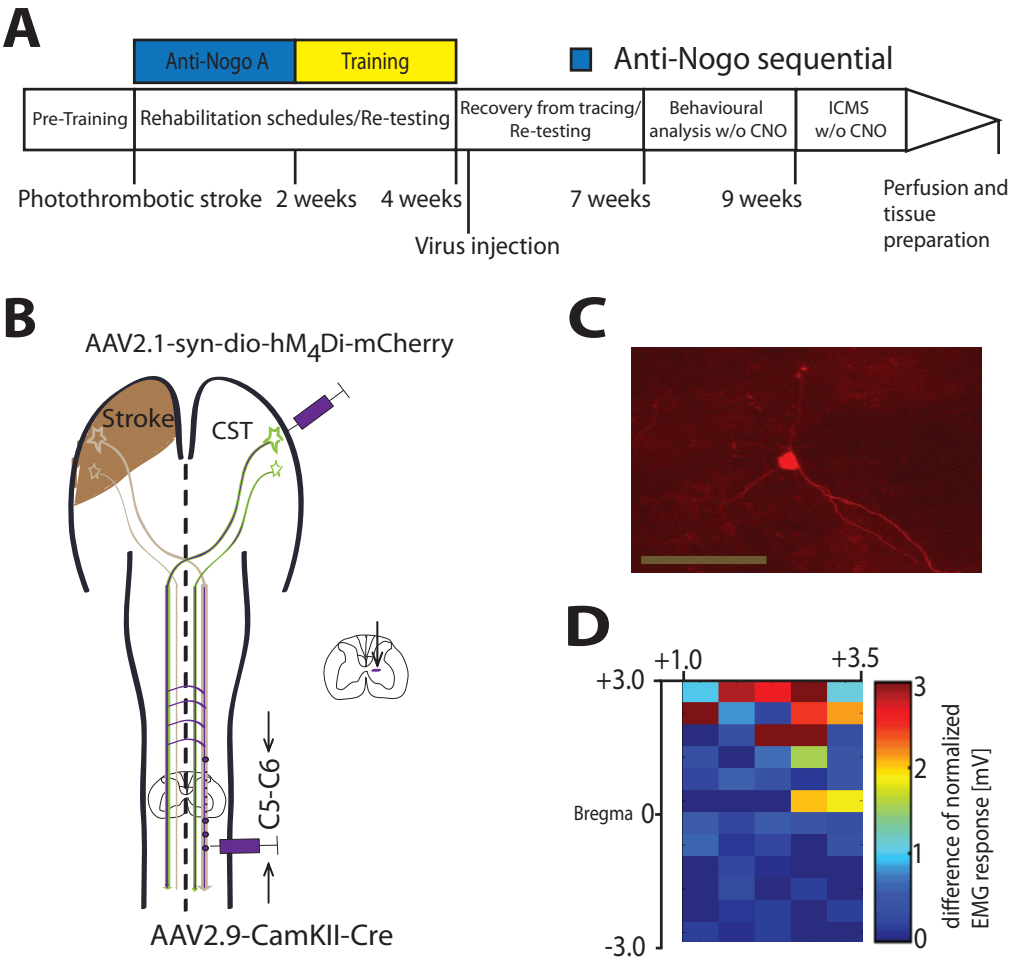

Fig. 5: Short term-reversible blockade of ipsilateral corticospinal fibers using DREADD. (A) The experimental schedule. (B) Schematic diagram of vector injections in the contralesional motor cortex (Cre-dependent AAV1-hsyn-dio-hM4Di-mCherry) and cervical spinal cord (AAV9-CaMKII-Cre). (C) mCherry positive pyramidal cell in layer 5 of contralesional motor cortex. Scale bar= 50 mum. (D) CNO application specifically reduced EMG responses in wrist recordings in the hM4Di group when premotor and rostral forelimb area of the contralesional motor cortex were stimulated. The heatmap shows the difference of EMG response due to CNO application for each stimulation point between the control, hM4Di/-Cre group and hM4Di/+Cre group.

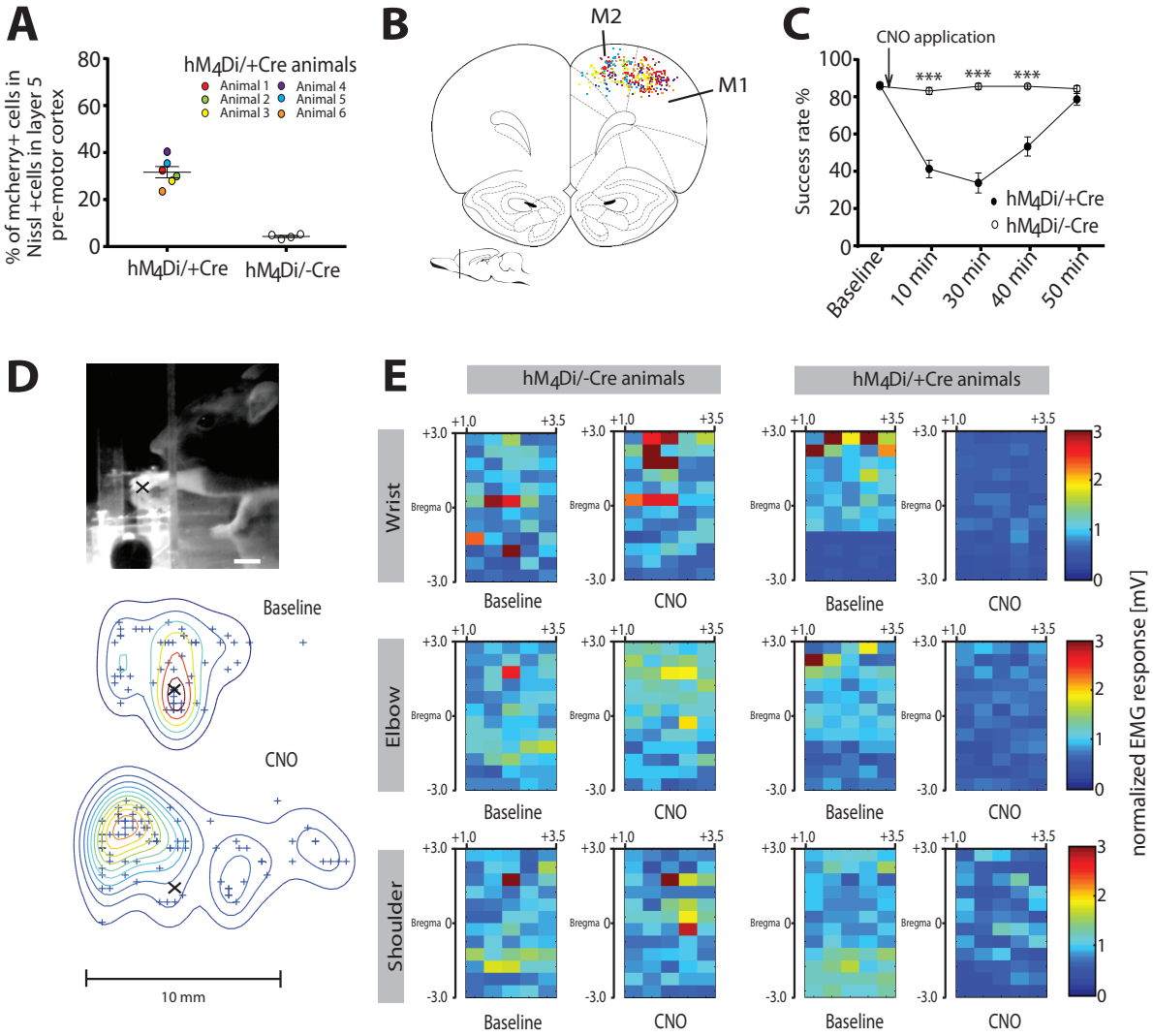

Fig. 6: Short term-reversible blockade of ipsilateral corticospinal fibers abolishes recovered grasping function and forelimb EMGs. (A, B) Quantification of mCherry positive cells in percentage of Nissl positive cells in layer 5 of contralesional pre- and motor cortex (M1), 3.7 mm anterior to bregma in the hM4Di/+Cre group (n=6) and the hM4Di/-Cre control group (n=4) (A) and the illustration of its location using Neurolucida reconstruction (B). (C) Activation of the DREADD receptor in the hM4Di/+Cre group induced a rapid, massive, but fully reversible impairment of grasping performance 10 – 40 min after CNO application; reaching success rates were unchanged in the control, hM4Di/-Cre CNO treated group over the same time frame. (D) CNO application disturbed fine motor function of closing the paw around the pellet in the hM4Di/+Cre group (p=0.02, K-S-Test): The figure shows the spatial probability densities for the location of hand closure relative to the sugar pellet during grasping at 'baseline' and after CNO application. x represents the position of the sugar pellet relative to the forelimb position during grasping. Scale bar = 10 mm. (E) CNO application leads to a decrease of EMG responses in the hM4Di/+Cre group (n=5) after ICMS of the contralesional motor cortex compared to ICMS stimulation at baseline and 30min after CNO application in control animals (n=3). Given are heatmaps of the cortical stimulation grid (60 stimulation points, 80 μA, +3 to -3 mm AP and 1 to 3.5 mm ML relative to bregma) whereby each stimulation point is color coded with the mean value of EMG response for a muscle group (wrist or elbow or shoulder) at this stimulation point (in mV) normalized to the mean of all stimulation points at baseline. Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-way ANOVA repeated measure followed by Bonferroni post hoc, asterisks indicate significances: $^{***}P < 0.001$.

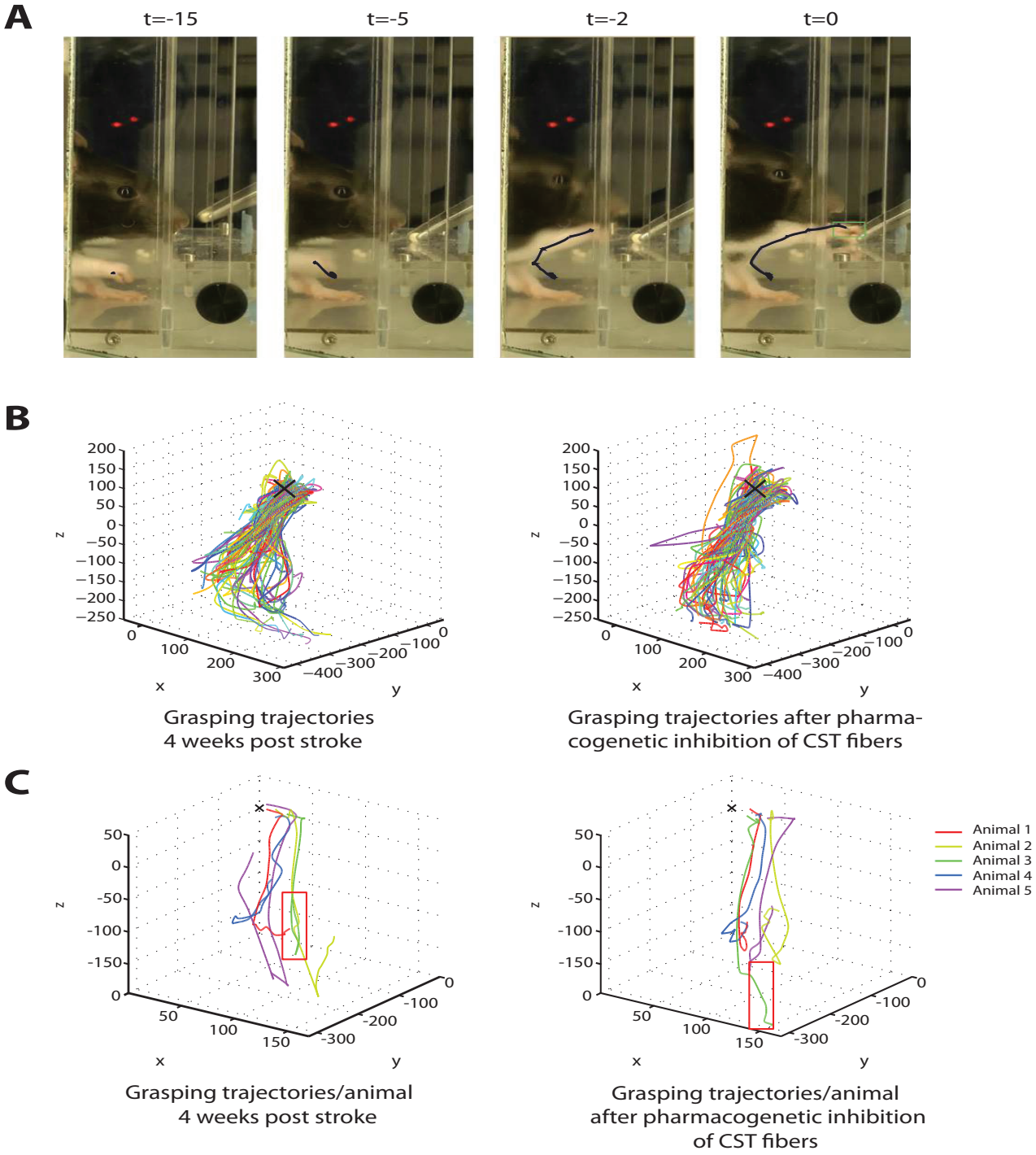

Fig. 7: Analysis of grasping kinematics before and during pharmacogenetic inhibition of the newly outsprouting CST fibers.(A) schematic illustration of backward tracking of the paw during the grasping process over 15 consecutive frames. (B) Overall grasping trajectories of animals in the Anti-Nogo/sequential-training rehabilitation group did not significantly change between 'baseline' (4 weeks post stroke at the end of the rehabilitation schedule) and after pharmacogenetic inactivation of CST fibers (p=0.65, K-S-test). The black cross indicates the sugar pellet position. (C) The mean trajectory of individual Anti-Nogo/sequential-training animals before and after pharmacogenetic inactivation of CST fibers. The red box indicates a significant difference of the motion trajectory of animal 3 (p=0.05, K-S-Test). However, over all animals this was not significant as shown in (b); asterisks indicate significances: $^*P < 0.05$

0(0 to 0.001); animal 2 0(0 to 0); animal 3 0(0 to 0.001); One-sample Wilcoxon signed rank test, Fig. 8C, Fig. 6E). In contrast, in 4 out of 5 hM4Di/+Cre animals the ICMS evoked EMG responses significantly decreased 30 min post CNO injection compared to baseline (for wrist 95% confidence interval was for animal 1 -0.002(-0.002 to -0.001); animal 2 -0.004(-0.005 to -0.003); animal 3 -0.002(-0.002 to -0.002), animal 4 0(0 to 0.001), animal 5 -0.012(-0.016 to -0.009), One-sample Wilcoxon signed rank test, Fig. 8D, Fig. 6E). Calculating the mean EMG response for every stimulation point in hM4Di/+Cre animals versus hM4Di/-Cre controls revealed a significant decline of EMG responses in wrist and elbow muscles in the hM4Di/+Cre group 30 min post CNO application compared to controls ($p < 0.05$, Mann-Whitney Test, Fig.6E). No significant abatement of EMG responses occurred in shoulder muscles of hM4Di/+Cre animals ($p = 0.4$, Mann-Whitney Test, Fig. 6E). CNO application resulted in the largest difference in EMG responses of wrist recordings when premotor and rostral forelimb areas of the contralesional motor cortex were stimulated in hM4Di/+Cre group compared to controls (Fig. 5D). These areas also expressed the highest concentration of mCherry expressing cells (Fig. 6A).

4.6 Discussion

Our study shows that in a rat model of large forebrain cortex strokes, timing of rehabilitative training relative to timing of a nerve fiber growth promoting therapy affects the recovery of lost motor function and the pattern of fiber sprouting. When rats received first Anti-Nogo-A immunotherapy followed by two weeks of specific, intense rehabilitative training forelimb function was almost fully restored (Fig. 1A, B) indicating a far more extensive recovery rate relative to stroke size than previously obtained by training (Adkins and Jones, 2005;Alaverdashvili et al., 2008; Starkey et al., 2011) or growth-promoting therapy alone (Lindau et al., 2014; Tsai et al., 2007). These animals not only outperformed the other rehabilitation groups in the single-pellet grasping task, they were also able to better transfer their regained skills to novel forelimb tasks (Starkey et al., 2011). The behavioral recovery was associated with crossing of CST fibers from the lesion-spared, intact motor cortex to the stroke-denervated side of the spinal cord. This observation is supported by various stroke and spinal cord injury models (Wiessner et al., 2003; Tsai et al., 2007; Maier et al., 2008; Garcia-Alias et al., 2009; Starkey et al., 2011) that relate midline-crossing corticospinal fibers to functional outcome. Our analysis shows that not only the quantity of newly out-sprouting corticospinal fibers is relevant but also their termination pattern: Intensive training, when applied too early, induced hyperinnervation and aberrant growth even beyond the grey/white matter boundary and into dorsal sensory laminae. Such widespread sprouting may results in wrong circuit connectivity involving cervical interneurons, V2a propriospinal neurons and motoneurons, thus impairing grasping function e.g. by co-activation of agonistic and antagonistic muscles (Asante and Martin, 2013; Azim et al., 2014).

Our data suggest the presence of critical time windows, during which the brain is most responsive to the application of growth promoting agents and to training-dependent plasticity. A correct, timed sequence of interventions is required to maximize the effectiveness of rehabilitative therapies after stroke. Suppression of the action of the endogenous growth inhibitory factor Nogo-A by immunotherapy in a first step may diminish constraints on lesion-induced structural plasticity through mechanisms such as neurotrophic factors, modified electrical properties of motoneurons, alteration in neuronal energy balance (Petruska et al., 2007) and recruitment of new circuits leading to hyperexcitability and prolonged responses to external stimuli (Murphy and Corbett, 2009). In analogy to development, many of these newly formed connections may be weak and imprecise. Training in a second step may then help to shape the spared and new circuits by selection and stabilization of functional connections and pruning of the

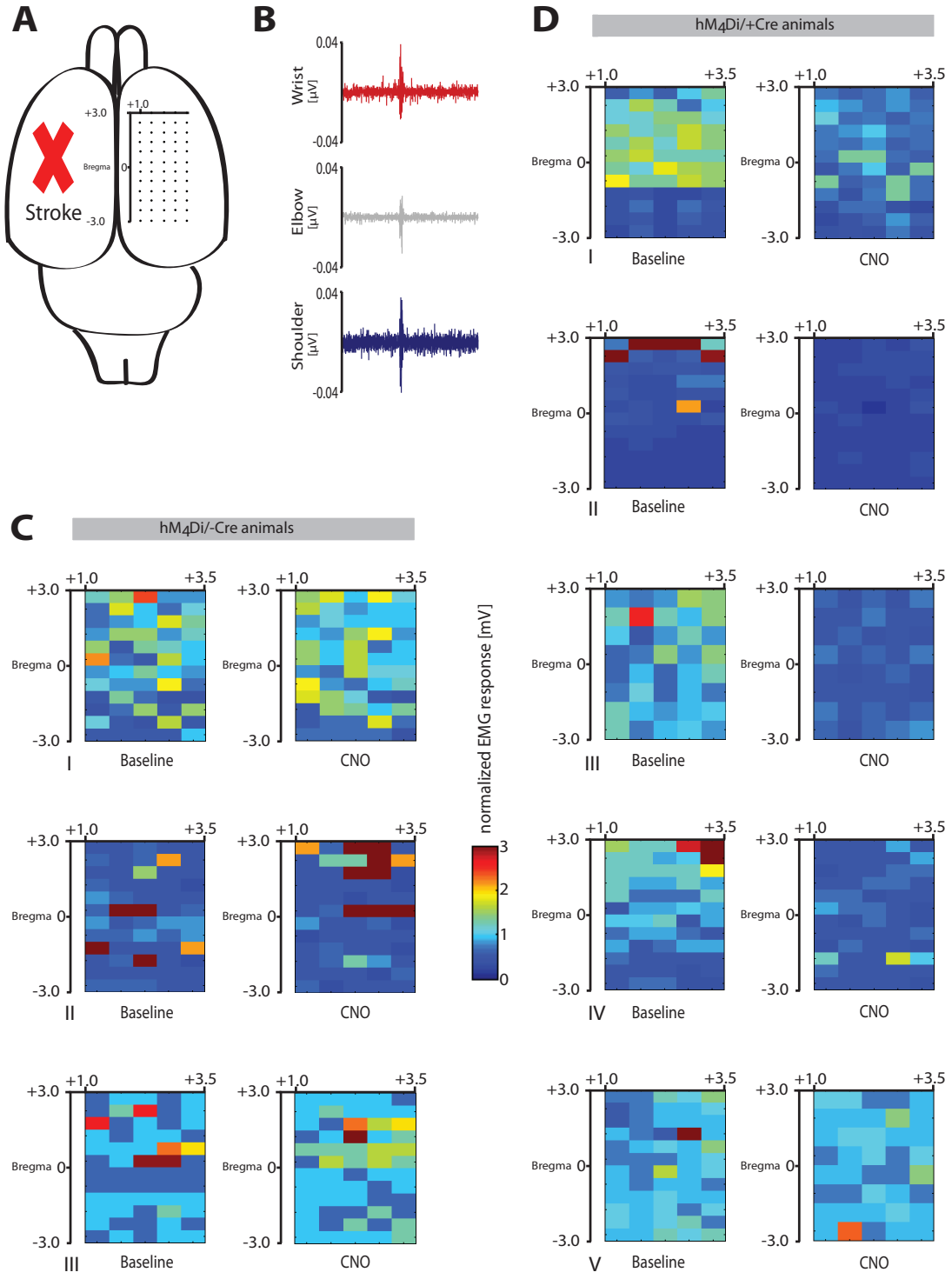

Fig. 8: Electrophysiological evaluation of reversible pharmacogenetic inhibition of CST fibers using intracranial microstimulation with forelimb EMG as a readout. (A) schematic illustration of the grid for ICMS (5 x 12 stimulation points, +3 to -3 mm AP and 1 to 3.5 mm ML relative to bregma) in the contralesional sensorimotor cortex. (B) Example of EMG recordings at wrist, elbow and shoulder muscles after intracortical microstimulation (ICMS) of the contralesional motor cortex. (C, D) Heatmaps at baseline and 30 min after CNO application for each animal in the two groups (control hM4Di/-Cre versus hM4Di/+Cre group) representing the maximal amplitudes of the EMG responses measured in wrist muscles at a distinct location of cortical stimulation relative to bregma. The value at each stimulation point (mV) was normalized to the baseline mean of maximal amplitudes at all stimulation points.

non-functional ones. This second step might involve Hebbian learning rules, in the sense that Hebbian plasticity redistributes synaptic strength to favor functionally relevant pathways that are coincidently active (Murphy and Corbett, 2009). The high degree of recovery of important, cortically controlled motor functions in rats with large ischemic strokes as demonstrated here points to a possible avenue to explore growth-inhibitor blockade in combination with rehabilitative training as a treatment strategy for humans with motor cortex stroke. Antibodies against Nogo-A are currently used in clinical trials in humans for amyotrophic lateral sclerosis, multiple sclerosis and spinal cord injury (www.clinicaltrials.gov). Careful consideration of rehabilitation onset times, in particular with regard to windows of sprouting and circuit plasticity but also vulnerability of the injured brain, and tailored training adapted to the type and extent of stroke and the patient's history will be essential for future approaches in the clinic (Dimyan et al., 2011; Krakauer et al., 2012).

4.7 Material and Methods

Experimental set-up

A total of $n = 70$ adult female Long-Evans rats (200-250g, 3-4 months of age, Janvier, France) were used in this study. Animals were housed in groups of three to four under a constant 12h dark/light cycle with food and water ad libitum. All experimental procedures were approved by the veterinary office of the canton of Zurich, Switzerland. They are in accordance with the Stroke Therapy Academic Industry Roundtable (STAIR) criteria (1999) for preclinical stroke investigations. The objective of this study was the examination of combined rehabilitative training with growth/plasticity enhancement by Anti-Nogo A antibody or control antibody treatment – sequentially and in parallel – in order to compare distinct rehabilitation schedules after a stroke for the restoration of skilled forelimb function. All animals received training in single pellet grasping followed by a photothrombotic stroke and the intrathecal applicaton of Anti-Nogo A antibodies or Ig G control antibodies delivered by osmotic mini pumps as described below. Only animals that revealed a severe lesion deficit two days post stroke surgery (less than 12% success rate in the single pellet grasping task compared to pre-stroke baseline levels) were included in this study. Animals were then randomized in four different rehabilitation groups. This experimental set-up was repeated in N=3 independent studies with $n = 12 - 16$ rats per cohort for behavioural and morphological analysis. As there was no statistically significant difference in the outcome of lesions, behavior and anatomy, the data shown here were pooled from all three studies. Animals were number-coded and investigators were always blinded to the treatment groups until the end of the data analysis.

Photothrombotic stroke and antibody treatment

Animals were anaesthetized with 3% isoflurane followed by a subcutaneous injection of a mixture of Hypnorm ($600\mu l/kg$ body weight, Janssen Pharmaceutics) and Dormicum ($3.75mg/kg$ body weight, Roche Pharmaceuticals). A photothrombotic stroke to unilaterally lesion the sensorimotor cortex corresponding to the preferred paw (see 'rehabilitative training' section) was introduced as previously described (Lindau et al., 2014). Briefly the animal was fixed in a stereotactic frame, the skull exposed through a midline incision, cleared of connective tissue and dried. A cold light source (Olympus KL 1500LCS, 150W, 3000K) was positioned over an opaque template with an opening for the light source (10 x 5mm) 5mm to -5mm anterior and 0.5mm to 5.5mm lateral to Bregma. Rose Bengal (13mg/kg body weight, 10mg/ml Rose Bengal in 0.9% NaCl solution) was injected in the femoral vein and after 2 min, the brain was illuminated through the intact skull for 10 min.

For constant delivery of the function blocking Ig G1 mouse monoclonal anti-Nogo-A antibody 11C7 (3 or $4.2 mg/ml$, gift from Novartis, Oertle et al., 2003) against an 18–amino acid Nogo-A peptide corresponding to the rat sequence amino acids 623 to 640 or the monoclonal control antibody anti-bromodeoxyuridine (BrdU; 3 or $4.2 mg/ml$, AbD Serotech), a fine intrathecal catheter (32 gauge) was placed in the subarachnoid space at lumbar level L2 after laminectomy and connected to an osmotic minipump (Alzet 2ML2; $5 \mu l/h$, $3.1 \mu g/\mu l$). For postoperative care all animals received analgetics (Rimadyl, $2.5 mg/kg$ body weight, Pfeizer) and antibiotics (Baytril, $5 mg/kg$ body weight, Bayer) for 3 days as well as a single subcutaneous injection of mannitol (20%, $17 mg/ml$, B. Braun) to reduce swelling of the cortex. After 2 weeks pumps and catheters were removed.

Rehabilitative training and testing

All animals were trained in the single pellet grasping task (Starkey et al., 2012) to assess fine motor control of the forelimb. Animals were placed in a Plexiglas box (34 x 14 cm) with two openings on opposite ends and had to grasp pellets (45-mg dustless precision pellets, TSE Systems Intl. Group). During testing sessions animals were given 20 pellets within a maximum time of 10 min. Grasping performance was scored as follows (Starkey et al., 2012): a trial, defied as the animal putting its paw through the grasping window to grasp a new pellet presented to the preferred side was scored as 1 (successful grasp) if the animal retrieved the pellet and brought it directly to its mouth. A score of 1 was also given if the animal required several attempts to grasp the pellet, without retracting the paw through the window and into the box, which was defined as the end of the attempt. A score of 0.5 was given if the animal successfully grasped the pellet but dropped the pellet inside the box. If the animal knocked the pellet off the shelf, the trial was scored as 0. The success rate was calculated as the percentage of retrieved pellets of the number of all trials. Animals were trained 3-4 weeks for baseline recordings before stroke. Only animals with a 60% or higher success rate at baseline were selected for the study. Two days post stroke surgery animals were re-tested in grasping to determine the lesion deficit and then randomized into the four rehabilitation groups ($'anti-Nogo-A/parallel'$, $'control/parallel'$, $'anti-Nogo-A/sequential'$, $'control/sequential'$): Here 'parallel' indicates the parallel training of forelimb function in single pellet grasping simultaneously to antibody application for 2 weeks while training was initiated for the animals in the 'sequential' groups after the completion of the antibody therapy and was maintained for another 2 weeks therefore ending 4 weeks post insult. Training consisted of 100 trials per session in 7 sessions/ week with the last 20 trials being scored for the success rate. Testing sessions were filmed (Panasonic HDC-SD800 High Definition Camcorder, 50 frames/s) for further analysis of grasping kinematics.

After completing the rehabilitative training schedules in the four rehabilitation groups all animals were exposed to novel tasks of grasping function such as the horizontal ladder test and the Montoya staircase (Montoya et al., 1991) in order to determine their non-task specific recovery levels of forelimb function. The irregular horizontal ladder crossing was conducted as reported previously (Maier et al., 2008): On three consecutive days three runs per animal were recorded (Panasonic HDC-SD800 High Definition Camcorder) and analyzed frame by frame (VideoReDo TV Suite, H 264, Drd Systems Inc.). The performance was scored as reported (Maier et al., 2008). The success rate was calculated by dividing the amount of correct steps by the number of total steps taken with the respective paw x 100 in the 3 test runs. The Montoya staircase was performed as described previously (Montoya et al., 1991): Pellets were presented on five different steps with 3 pellets per step to the animals placed into the staircase box for 10 min. As the level of difficulty for reaching the pellets from the different steps changed, the amount of eaten pellets from each step was multiplied by the number of the step. Then all products were

added to the 'sum of eaten pellets' (='eaten pellets step 1' x 1 + ...+'eaten pellets step 5' x 5). The success rate in the test ('sum of eaten pellets' x 100) was normalized to the maximum possible score (45) in this test.

Anterograde tracing of the intact corticospinal tract (CST)

After the completion of the behavioural training and testing, the intact contralesional motor cortex of animals in all 4 rehabilitation groups ($'anti-Nogo-A/parallel'$ n=10, $control/parallel$ n=8, $'anti-Nogo-A/sequential'$ n=10, $'control/sequential'$ n=8) was traced anterogradely with Biotinylated Dextran Amine (BDA, 10,000 molecular weight, 10% solution in 0.01 M PBS, Invitrogen). 10 stereotactic injections (200 nl each) were made through the intact dura using a 35-gauge, $10\mu l$ syringe (World Precision instruments) with a flow rate of $6nl/s$ controlled by an electrical pump (World Precision instruments). The injection coordinates for the motor cortex were: 2 mm anterior to bregma (AP), 3 mm lateral to bregma (ML); 2.5 AP, 3.5 ML; 1.5 AP, 3.5ML; 1.5 AP, 2.5 ML; 2.5 AP, 2.5ML; -2.0 AP, 2.0 ML; -1.5 AP, 2.5 ML; -2.5 AP, 2.5 ML; -2.5 AP, 1.5 ML; -1.5 AP, 1.5 ML. All injections were at 1.5 mm depth and the syringe remained in place for 2 min after completion of each injection.
Three weeks after BDA injections animals were anaesthetized (pentobarbital, $450mg/kg$ body weight i.p., Abbott Laboratories) and perfused transcardially with 100 ml Ringer solution (containing $100000IU/l$ heparin (Roche) and 0.25% NaNO2) followed by 300ml of a 4% phosphate-buffered paraformaldehyde solution, pH 7.4. Brains and spinal cords were dissolved and cryoprotected in a phosphate-buffered 30% sucrose solution for cryostat sectioning in $40\mu m$ thick sections before being stained by on-slide processing using the nickel-enhanced DAB (3,3'-diaminobenzidine) protocol (Vectastain ABC Elite Kit, Vector Laboratories; 1:100 in Tris-buffered saline plus TritonTM X-100).
CST fiber growth in response to stroke was evaluated at spinal cord levels C2-Th1. Fibers crossing the spinal cord midline were counted at 20x magnification and branching of these fibers was evaluated at 4 defined distances from the midline in the grey matter using a virtual grid (D1-D4, Fig. 2a), with each line $125\mu m$ apart. To correct for variations in BDA labelling, we normalized the data to the number of BDA-labelled axons in the intact CST for each animal (CST axons: counted in five rectangular areas ($4000\mu m^2$) and extrapolated to the total area of the CST ($0.1144mm^2 \pm 0.01$) per slice in two sections at spinal cord level C2 and C5. Results are expressed as newly out-sprouting fibers divided by the number of labelled fibers in the intact CST for each animal.

Analysis of sprouting patterns in the cervical spinal cord

Mosaic images of 3 sections at spinal cord level C4 of each animal which received BDA tracing were obtained (Zeiss Axioskop 2MOT with motorized stage, 20x magnification) for analysis of distinct sprouting patterns according to the four different rehabilitation groups. Each image was labelled with 3 landmarks (midline of the spinal cord, lateral tip of the ventral horn, dorsal white-grey matter border of the dorsal horn) using a standard template of the cervical level C4. Images were then warped to bring the landmark points into correspondence and compensate for the overall deformation. Fibers were detected by computing the probability of boundary (Maire et al., 2008) and using morphological operators to compensate for noise. Thereafter, we trained detectors to localize fiber crossings. 100 exemplary crossings were cropped from images, clustered into ten groups (agglomerative clustering with Ward's method), and used as training samples for linear Support Vector Machines. The discriminative classifiers for each of the ten groups provided a linear filter. Images were then convolved with this filter bank and non-maximum suppression was performed to detect fiber crossings. The combination of

unsupervised clustering and discriminative classification was necessary to render the approach computationally feasible for large-scale experimentation. A similar approach was followed for bouton detection. Again 100 training samples were gathered and clustered into ten groups before training linear filters using Support Vector Machines.

MRI images for stroke volumetry

Prior to ex vivo imaging for determination of lesion size the brain tissue samples were placed in 15 ml falcon tubes filled with perfluoropolyether (Fomblin®Y-LC 80, Solvay Solexis, Bollate, Italy). The MR measurements were performed using a 4.7T (200 MHz) small animal MR system (Bruker BioSpin GmbH, Ettlingen, Germany), equipped with a volume resonator operating in quadrature mode for excitation and a four-element phased array surface coil for signal reception. T2-weighted images, in total 15 coronal slices of 0.9mm thickness with an interslice distance of 1.10mm covering most of the brain, were acquired using a TurboRARE sequence with the following parameters: field of view = 20 x $20mm^2$, matrix dimension = 200 x 200, yielding an in-plane voxel dimension of 100 x $100\mu m$, repetition time = $3930ms$, echo time = $28ms$, effective echo time = $84ms$, RARE factor = 8, number of averages = 15.

After ex vivo MRI, the brains were removed from Fomblin and placed again in PFA. To quantify the degree of the lesion, automatic shape analysis was employed. A compositional registration and warping algorithm was developed to extract the shape of the lesion and measure its size. Following the approach of Yarlagadda and Ommer (Yarlagadda and Ommer, 2012), probability of boundary was computed for MRI image stacks and edge pixels were grouped into candidate compositions. Thereafter, similar compositions were re-detected to find correspondences between both brain hemispheres and to identify a symmetry plane. This registration of compositions against a large degree of background clutter was based on the max-margin discriminative chamfer matching with explicit modeling of background accidentalness (Eigenstetter et al., 2012). As described (Eigenstetter et al., 2012), the compositional shape matching and the max-margin chamfer regularization are significantly more robust with respect to noise, occlusion, or accidental distortions than a direct registration of edge pixels. As a result of the compositional shape matching, both brain hemispheres were identified and the lesion, which is an abnormality that is present in only one hemisphere, was detected and quantified in size.

Histological analysis of stroke volume

After ex vivo MRI imaging, rat brains fixed in 4% PFA were placed in a phosphate-buffered 30% sucrose solution at 4°C before being cryoprotected. The brains were embedded in Tissu-Tek®O.C.T.TM and frozen in isopentane (Sigma) at −40°C. Brains were cut coronally on a cryostat in $40\mu m$ sections and collected on slides (SuperFrost®). The frozen sections were dried at room temperature, rehydrated and immersed in 0.5% cresyl violet (2 min) for Nissl staining. After washing in water, the sections were dehydrated in graded alcohols, cleared in xylene and cover-slipped with Eukit®mounting medium. The lesion volume of all four rehabilitation groups was evaluated by 3D reconstruction of every 10th brain section, using Neurolucida 8.0 (MicroBrightField).

TeNT experiment

For long-term reversible blockade of midline crossing fibers originating from the intact CST we combined two viral vectors being part of a Tet-on system as described (Kato et al., 2011; Kinoshita et al., 2012): Animals were anaesthetized as mentioned previously for the stroke lesion and a craniotomy was performed exposing the contralesional pre and sensorimotor cortex:

12 injections (200nl each) of the Tet-on transactivator AAV2.2-CMV-rtTAV16 (1.6 x 10e13 copies/ml) were made as described above for the anterograde BDA tracing using the same coordinates plus two coordinates in the premotor cortex (Fig. 4B): 2.2 AP, 2.5 ML; 2.2 AP, 3.5 ML. 2 days later, a minimal invasive laminectomy at spinal level C5-C6 was performed. 11 x 120nl of the highly efficient retrograde gene transfer (HiRet) vector HiRet-TRE-EHGFP-eTeNT (7.2 x 10e11 copies/ml)(n=7) or the control vector HiRet-TRE-EGFP (9.0 x 10e11 copies/ml) (n=4) were injected in the denervated cervical hemi spinal cord (Fig. 4B) 0.7mm lateral to the midline and 1.2mm below the spinal cord surface using a 35-gauge, 10ml syringe (Hamilton, BGB Analytik) driven by an electric pump (World Precision Instruments) with a flow rate of 6nl/s. Each injection was made in 2 steps of 60 nl keeping the syringe in place for 3min between steps. After 1 week of recovery animals were re-trained and re-tested in the single pellet grasping task for 2 weeks (Fig 4A) before doxycycline administration. For initiation doxycycline (Doxycycline hyclate, Sigma-Aldrich) was applied via i.p. injection (10mg/kg body weight in 0.9% NaCl) followed by oral administration (2mg/ml in 5% sucrose) during the course of the experiment. Oral intake of the doxycycline/sucrose solution in combination with body weight was checked on a daily basis. Doxycycline was applied twice for 3 weeks with a break of 2 weeks in between and animals were continuously re-assessed in the function of their impaired paw. At the end of the experiment animals were anaesthetized and transcardially perfused as described above (section 'Anterograde labeling of the CST'). Coronar cortex sections ($40\mu m$ slice thickness) were examined for the distribution of EGFP-positive neurons with anti-GFP immunohistochemistry: Immediately after cryo-sectioning slices were blocked in Tris-NaCl-blocking buffer and 0.1% Triton (TNB, TBST) for 1h at room temperature and incubated over night at 4°C in TNB, TBST and the primary antibody green fluorescent protein (GFP) (rat monoclonal antibody, 1:1000; Nacalai). The biotinylated Goat Anti-Rat immunoglobulin-G (Ig G) (1:300; Jackson IR) secondary antibody in PBS was applied for 2 h at room temperature. The sections were washed in PBS and incubated in Alexa 488 Streptavidin (1:1000; Jackson IR) and fluorescent Nissl 640/660 (1:500; Neurotrace Invitrogen) in TNB. Images were acquired with a confocal microscope (Leica TCS SP2) with respective green (FITC) and infrared (Cy5) excitation or emission filters at 20x magnification. The percentage of GFP-positive cells of Nissl positive cells in layer 5 of the sensorimotor cortex was counted in 5 adjacent sections 3.7mm ($\pm$ 200 μm) anterior to bregma (Fig. 4D,E).

DREADD experiment

For short-term reversible blockade of midline crossing fibers originating from the intact CST we used viral mediated- expression of an engineered G protein-coupled receptor (Gi/o-coupled human muscarinic M4 designer receptor exclusively activated by a designer drug, hM4Di) that is activated by an otherwise pharmacologically inert ligand, clozapine-N-oxide (CNO) (Conklin et al., 2008; Alexander et al., 2009; Ferguson et al., 2011). In order to selectively express hM4Di in midline crossing fibers coming from the intact CST we applied a Cre-dependent approach: After completion of the rehabilitative training n=6 animals were injected with AAV2.1-hSyn-dio-hM4D(Gi)-mCherry vector (UNC Vector Core, the University of North Carolina at Chapel Hill) in the contralesional pre- and sensorimotor cortex followed by injection of an AAV2.9-CamKIIα.-Cre vector (Penn Vector Core, Philadelphia) in the denervated cervical hemi spinal cord at spinal cord level C5-C6 (Fig. 5B) as described above ('TeNT experiment'). Control animals (n=4) just received injections of the AAV2.1-hSyn-dio-hM4D(Gi)-mCherry vector in the cortex without application of the Cre-Vector in the spinal cord. 3 weeks post surgery animals were video-taped (100 frames/s, GenieTM Teledyne Dalsa) in single pellet grasping as 'baseline' recording followed by i.p. injection of CNO (1.2mg/kg body weight; Enzo Life Sciences). Animals were immediately put back into the grasping box and grasping performance

was recorded for the next 50 min for further analysis ('Analysis of grasping performance' below). Each animal was tested for 3 independent trials in single pellet grasping at baseline and under CNO application. Results are expressed as mean of these 3 trials per animal. The same animals were then used for intracortical microstimulation (ICMS) described below. After completion of all experiments coronar cortical sections ($40\mu m$) were stained with fluorescent Nissl 640/660 (1:500; Neurotrace Invitrogen) in PBS and examined for the distribution of mCherry positive neurons in the pre-and sensorimotor cortex by taking images with a confocal microscope (Leica TCS SP2) with respective red (TRITC) and infrared (Cy5) excitation or emission filters. Analysis of mCherry positive cells in relation to Nissl positive cells was undertaken as above (see section 'TeNT experiment').

Analysis of grasping performance

Based on an automated unsupervised tracking, and a discriminative machine learning approach we studied the overall grasping kinematics as well as the process of closing the paw.
To analyze the grasping kinematics we first recognized the grasping event and localized it in space and time. Therefore, the sugar pellets were detected using a holistic recognition algorithm (Monroy and Ommer, 2012). The forelimb was tracked in both temporal directions (forward and backward) from the moment it came close to the pellet. As there was significant background clutter and no manual initialization was provided for the tracking, we utilized a generic compositional recognition and tracking approach (Ommer et al., 2009). This method provided spatiotemporal grasping trajectories which were brought into the same reference frame by first registering the scene of each camera shot to compensate for overall variations in magnification, alignment, etc. For each experiment and time point (Baseline, 30min CNO application, and 50min CNO application) we thus obtained average trajectories per animal and performed a pairwise agglomerative clustering with Ward's method which provided a grouping of trajectories into two clusters corresponding to hM4Di/+Cre and hM4Di/-Cre animals. We then performed a discriminative analysis to measure the probability of misclassification.
The process of closing the paw upon grasping was analyzed by a discriminative classification approach. Therefore the paw posture and its change were jointly represented using a non-parametric model (Ommer et al., 2009), which comprises appearance, shape, and motion. Then a classifier was trained on these representations to separate functional from non-functional grasps at baseline and 30min. Classification yielded a separating manifold and a projection into a low-dimensional subspace, which best discriminated samples from baseline and 30min. Projecting the 50min data into this subspace, yielded a contrasting measure between baseline/30min and baseline/50min. We then compare these measures for hM4Di/+Cre and hM4Di/-Cre control animals based on a KS-test.

Intracortical microstimulation

For intracortical microstimulation hM4Di/+Cre and hM4Di/-Cre control animals were anaesthetized with a subcutaneous mixture of ketamine (50mg/ml, 7mg/ kg body weight, Streuli Pharma) and xylazine (20mg/ml, 5mg/kg body weight, Streuli Pharma) plus a single injection of mannitol (20%, 17ml/kg, B. Braun). The forelimbs were shaved for better visibility of muscles and the rat was mounted in a stereotactic frame (Emmerick et al., 2003). A craniotomy was performed on the contralesional side exposing the entire motor cortex (4mm to -4mm AP, 4mm lateral relative to bregma). Using bregma as a landmark electrode penetrations were made perpendicular to the pial surface (depth 1.3mm) tracing an rectangular exploration grid of 5 x 12 stimulation points localized from 3mm to -3mm AP and 1 to 3.5mm ML relative to bregma with a distance of $500\mu m$ for each stimulation point (Fig. 8). Forty-five-millisecond trains of

0.2ms biphasic pulses at 333 Hz (Lindau et al., 2014) were delivered through a glass isolated platinum/tungsten stimulation electrode with an impedance of 0.5 – 1MΩ (Thomas Recording). EMG recordings from the ipsilateral forelimb (M. extensor digitorum for wrist, M. biceps and triceps for elbow, M. trapezius for shoulder) were used as readouts. The same points within the exploration grid were stimulated twice with a current of $80\mu A$ insuring a stable response (Brus-Ramer et al., 2007; Petruska et al., 2007; Soleman et al., 2012): First for 'baseline' stimulation, then again, 30 minutes after i.p. injection of CNO (1.2mg/kg body weight), to determine electrophysiological modifications of corticospinal signal transmission under CNO. The EMG signal was amplified, filtered, digitized and visualized via PowerLab (AD instruments). The EMG data was subsequently transferred to Matlab and the maximum of the EMG-amplitude was detected at each stimulation point and for all joints. Heatmaps represent the maximal amplitudes of the EMG responses in a joint at a distinct location of cortical stimulation relative to bregma. Maximal amplitudes at all stimulation points at baseline or under CNO dosage were normalized to the mean of maximal amplitudes at all stimulation points at baseline. For the calculation of the difference of EMG responses due to CNO application between the control, hM4Di/-Cre group and hM4Di/+Cre group the following formula was used for each stimulation point: hM4Di/-Cre ((meanCNO - meanbaseline)/meantotal) - hM4Di/+Cre ((meanCNO - meanbaseline)/meantotal), meantotal= mean of maximal amplitudes at all stimulation points at baseline.

Statistical analysis

All data are expressed as mean ± standard error of the mean (s.e.m.). For comparing the behavioural and anatomical reorganization of the four rehabilitation groups, a two-way ANOVA followed by Bonferroni's post hoc test was used. In all these experiments differences between the rehabilitation groups were significant for the chosen size of the cohort, thus justifying samples size. Whenever two treatments were compared at one time point, Student's t-test (two-tailed, unpaired) was used. For correlation analysis between behavioural recovery and out-sprouting fibers or stroke volumetry Spearman correlation was applied. The Mann-Whitney Test as well as the Wilcoxon Signed Ranks Test were used as non-parametric tests to test for statistical significant difference of ICMS data between groups as well as between conditions within each animal. The level of significance was set at $^{*}P < 0.05$,$^{**}P < 0.01$, $^{***}P < 0.001$.

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Chapter 5

Different rehabilitation schedules induce distinct spatio-temporal expression patterns of growth-promoting genes after stroke

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Abstract

Nervous system injury such as stroke induces the expression of growth promoting genes and neurotrophic factors as part of the intrinsic molecular repair machinery that orchestrates plastic processes determining the location and degree of neuronal rewiring. However, how external stimuli such as Anti-Nogo immunotherapy and rehabilitative training influence internal repair processes is not well understood. Here we performed a candidate-based screening for the induction of growth promoting genes and neurotrophic factors in tissue of rats with different rehabilitation paradigms after a large photothrombotic stroke in the sensorimotor cortex. Rats either received no rehabilitation ('spontaneous recovery' group) or intensive rehabilitative training of the forelimb concurrently ($'Anti-Nogo-A/parallel'$ group) or after the application of Anti-Nogo-A immunotherapy ($'Anti-Nogo-A/sequential'$ group) or control Ig G antibody ($'control/parallel'$ or $'control/sequential'$ group). Tissue for analysis of induced mRNA levels of growth-promoting genes and neurotrophic factors was extracted at distinct areas adjacent and remote from the stroke core in the brain and spinal cord at 7 and 28 days after stroke. We found distinct temporal and spatial expression patterns of growth-promoting genes depending on the rehabilitative paradigm: *BDNF* mRNA levels were highly up-regulated in the penumbra and the ipsilesional cervical hemi spinal cord of the $'Anti-Nogo-A/sequential'$ group. The excellent functional recovery rate of this group correlated with *BDNF* levels in the spinal cord. Only spinal cord tissue of the $'Anti-Nogo-A/sequential'$ group was significant for increased levels of *STAT3* - a pathway associated with enhanced axonal regeneration after CNS injury. In contrast, brain and spinal cord tissue of the $'Anti-Nogo-A/parallel'$ group which had displayed the poorest functional outcome, revealed increased mRNA levels of *GFAP* and *Synapsin Ib* in areas of the brain and spinal cord indicating gliosis as well as aberrant and excessive synaptogenesis.

In summary, this study provides first insights into molecular mechanisms which might underlie structural and functional recovery and indicate a critical threshold when rehabilitative training could be successful. Furthermore, data suggest that *BDNF* is a key player mediating efficiency of optimal rehabilitation paradigms.

5.1 Introduction

Within the last 10-15 years the old paradigm of the adult CNS as a stable and static structure has been replaced by new concepts showing a high capacity of the CNS for neuronal remodeling and repair after stroke (Schwab et al., 2010). Recent studies in humans and animal models revealed rewiring of neuronal circuits, neuro- and synaptogenesis, spine turnover (Brown et al., 2007) and increased neuron - to glia interaction contributing to synapse remodeling and circuit re-shaping (Wake et al., 2009) in areas adjacent to and remote from the stroke lesion (Murphy and Corbett et al., 2009; Takatsuru et al., 2009). Although the exact details for the underlying molecular and cellular mechanisms are lacking, there is evidence that the basis of these 'hardware' changes are intrinsic molecular repair algorithms with precisely timed and spatially controlled gene induction and repression comparable to rules held during the development of the nervous system and experience-dependent plasticity (Murphy and Corbett, 2009).
Although the synthesis of most mRNAs and their respective proteins decreases following cerebral ischemia (Kleihues and Hossmann, 1971; Kiessling et al., 1986) more than 90 different genes have been shown to be acutely induced, generally with an early peak within minutes or hours of onset of ischemia and a rapid return to normal or subnormal levels. Whether the early transient increase in gene expression during the initial post ischemic hours is related to outcome remains unknown (Johansson et al., 2000). Carmichael et al., 2005 were the first showing the induction of sequential waves of growth- promoting and inhibiting genes in the peri-infarct cortex for the initiation, maintenance and termination of axonal sprouting using a model of stroke in the rat somatosensory barrel cortex. Growth-promoting genes mediate growth cone membrane signaling events, transcriptional control in the regenerating neuron, cytoskeletal reorganization and axonal extension. They include immediate early genes such as *c-fos*, *junB* and *c-jun* as well as *GAP43*, *CAP23*, *MARCKS*, members of the stathmin family, *Tα1 tubulin*, *L1*, *p21/waf1*, and *SPRR1* (Carmichael et al., 2005).
Furthermore, several neurotrophic factors are induced in an early response to stroke (Mattson, 2008; Carmichael, 2012): The up-regulation of the brain-derived neurotrophic factor (*BDNF*), nerve growth factor (*NGF*) and neurotrophin 3(*NT-3*) as well as fibroblast growth factor (*FGF2*) and insulin-like growth factor (*IGF-1*), epidermal growth factor (*EGF*) and glial cell line-derived neurotrophic factor (*GDNF*) have been described (Carmichael, 2012). In addition, each neurotrophic factor species depicts a specific temporal and cellular expression pattern (Abe et al., 2000): While GDNF is mainly distributed in neurons, astroglia induce CNTF expression and the vascular endothelial growth factor (*VEGF*) gene expression is detected in both cell types - neurons and glia- after stroke.
For optimal axonal sprouting and neuronal rewiring not only the induction of growth-promoting but also the repression of growth-inhibiting molecules is crucial. Three general classes of axonal growth inhibitors are found in the CNS: myelin associated proteins (NogoA, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein), extracellular matrix proteins (e.g. chondroitin sulfate proteoglycans) and repulsive cues for growth cones usually described as key players during development (e.g. ephrins, semaphorins). The expression of the chondroitin sulfate proteoglycans aggrecan, phosphacan and versican appears relatively delayed after stroke compared to the early and middle phase of the growth-promoting gene expression (Carmichael et al., 2005; Carmichael et al., 2012).
Besides the distinct waves of temporal expression patterns, the spatial distribution of growth-promoting and inhibiting genes plays a central role to induce neuronal self-repair processes: E.g. in the peri-infarct cortex, axonal sprouting takes only place close to but in distance to the glial scar. Within the glial scar which represents the wall separating the stroke core from the surviving per-infarct tissue, both, growth-promoting and growth inhibiting factors, are expressed while the peri-infact cortex represses the induction of growth inhibiting molecules. Vice

versa, mRNA levels of neurotrophins are largely enhanced in the growth-permissive penumbra and suppressed in the stroke core (Lanfranconi, 2011).

Together, the precise interplay of timed induction of growth promoting and the repression of growth inhibiting genes at distinct locations within and apart from the stroke core orchestrate plastic processes after stroke which are the fundament for spontaneous recovery. They define a time window in which the central nervous system is vulnerable but can also be further shaped through enhanced rewiring processes by external stimuli. Such external stimuli represent rehabilitative training, pharmacological intervention and electrical devices to stimulate neuronal repair. The challenge for neuro-rehabilitative strategies remains to capitalize the vulnerable period and enhance the internal remodeling process by external intervention.

Aim of the study

In chapter 4 we combined Anti-Nogo immunotherapy with rehabilitative training after stroke, whereby rehabilitative training was either applied concurrently or after completion of the immunotherapy. Control groups either received an inactive control body with early or delayed training after stroke. The application of the four rehabilitation paradigms resulted in very diverse levels of functional recovery for forelimb fine motor skills rising the question of different underlying molecular mechanisms. The objective of this study is the examination of distinct induction patterns for growth-promoting genes and neurotrophic factors in these four rehabilitative treatment groups in rats with stroke. This candidate-based screen may provide first insights in the efficiency of rehabilitative schedules and may help to understand the molecular basis of one rehabilitation strategy excelling the others.

5.2 Material and Methods

Animals

A total of n= 30 adult female Long-Evans rats (200-250g, 3-4 months of age, Janvier, France) were used for tissue analysis either after photothrombotic stroke and spontaneous recovery or rehabilitation or in naive animals as controls. Animals were housed in groups of three to four under a constant 12h dark/light cycle with food and water ad libitum. All experimental procedures were approved by the veterinary office of the canton of Zurich, Switzerland. They are in accordance with the Stroke Therapy Academic Industry Roundtable (STAIR) criteria (1999) for preclinical stroke investigations.

Experimental Set-up

In the first cohort of animals a photothrombotic stroke was introduced over the sensorimotor cortex as described before (Chapter 4, Lindau et al., 2014) and tissue from the brain and spinal cord was extracted for RNA extraction 7 or 28 days after the stroke surgery. For the second cohort animals were trained in the single pellet grasping task (Metz and Whishaw, 2000) before unilaterally lesioning the sensorimotor cortex corresponding to the preferred paw and before implanting osmotic mini pumps for the intrathecal applicaton of Anti-Nogo A antibodies or Ig G control antibodies as described (Chapter 4, Material and Methods). The function blocking Ig G1 mouse monoclonal anti-Nogo-A antibody 11C7 (3 or 4.2 mg/ml, gift from Novartis, Oertle et al., 2003) against an 18–amino acid Nogo-A peptide corresponding to the rat sequence amino acids 623 to 640 or the monoclonal control antibody anti-bromodeoxyuridine (BrdU; 3 or 4.2 mg/ml, AbD Serotech) were delivered for 2 weeks post stroke. After re-testing in the single

pellet grasping task to assess the lesion deficit animals were randomly distributed to the four rehabilitation groups as described in Chapter 4: Animals either received rehabilitative training in grasping of the impaired forelimb either concurrently to Anti-Nogo antibody immunotherapy or Ig G control antiody application ($anti-Nogo-A/parallel$ group; $Control/parallel$ group) or sequentially for 2 weeks after the completion of the immunotherapy ($anti-Nogo-A/sequential$ group; $Control/sequential$ group). Brain and spinal cord tissue was extracted for further analysis 4 weeks after stroke after the completion of all rehabilitation schedules.

RNA extraction and cDNA synthesis

For tissue extraction animals were anesthetized with 3% isoflurane before decapitation. The brain and spinal cord were quickly removed while dissecting on ice and put into fluid nitrogen for storage at $-80°C$. Brains and spinal cord were then transferred to $-20°C$ using the RNAlater®-ICE Frozen Tissue Transition Solution (Ambion®) to keep the RNA in the tissue stable. Brains and spinal cord were dissected on ice under a microscope.
To determine the expression levels for growth-promoting genes in the different tissue subsets we removed the stroke core (labelled as 'stroke i'), which was identified by a typical glial scar and cystic formation, as well as tissue in the contralesional cortex (labelled with 'stroke c') at the same rostrocaudal position (5mm to -5mm anterior and 0.5mm to 5.5mm lateral to Bregma). According to Keyvani et al., 2002 we removed a frame of tissue within a distance of 2.5mm to the borders of the stroke core (labelled with 'penumbra i') for the analysis of the penumbra region as well as the corresponding tissue in the contralesional hemisphere (labeled with 'penumbra c'). We also extracted tissue of the visual cortex (2mm broad band from the very caudal part of the cortex) ispi- and contralaterally to the stroke (labelled with 'visual cortex i' and 'visual cortex c').
For the analysis of the spinal cord tissue we first dissected the ispi- and contralesional hemi-spinal cord along the midline from spinal cord level C4 to C6 (enlargement of the cervical spinal cord) and then extracted in a second step the grey matter under the micrsocope. All tissue extraction was performed on ice and all dissected tissue was directed transferred to QIAzol Lysis Reagent (Qiagen, Hilden, Germany), homogenized using micropistles while chloroform was added to perform a phenol-chloroform extraction for RNA purification. For the following steps we used the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer' s instructions with additional on-column DNase I digestion during RNA purification. The final concentration of the isolated RNA was adjusted to 50 ng/μl. For reverse transcription, equal amounts of total RNA were transformed by using oligo(dT) primers and M-MLV reverse transcriptase (Promega) (Pernet et al., 2013).

Semi-quantitative real time RT-PCR (qRT-PCR)

We used qRT-PCR measurements in different tissue (stroke core, contralesional 'stroke area', penumbra, contralesional penumbra 'area', ispi- and contralesional spinal cord as well as ispi- and contralesional grey matter of the cervical spinal cord C4-C6) as described by Pernet et al., 2012. Relative quantification was calculated using the comparative threshold cycle (DD_{CT}) method. cDNA levels were normalized to *Gapdh* (reference gene) and a control sample (calibrator set to 1) was used to calculate the relative values.
The PCR amplification efficiency was established for each gene from the slope of the calibration curve according to the equation: $E = 10(\_1/slope)$ (Pfaffl, 2004). We performed triplicates for each reaction and at least $n = 3-4$ mice per condition were analyzed. The cDNAs corresponding to 10ng of total RNA were amplified with the following specific primers designed to span intronic sequences or cover exon–intron boundaries: glyceraldehyde-3-phosphate dehydrogenase

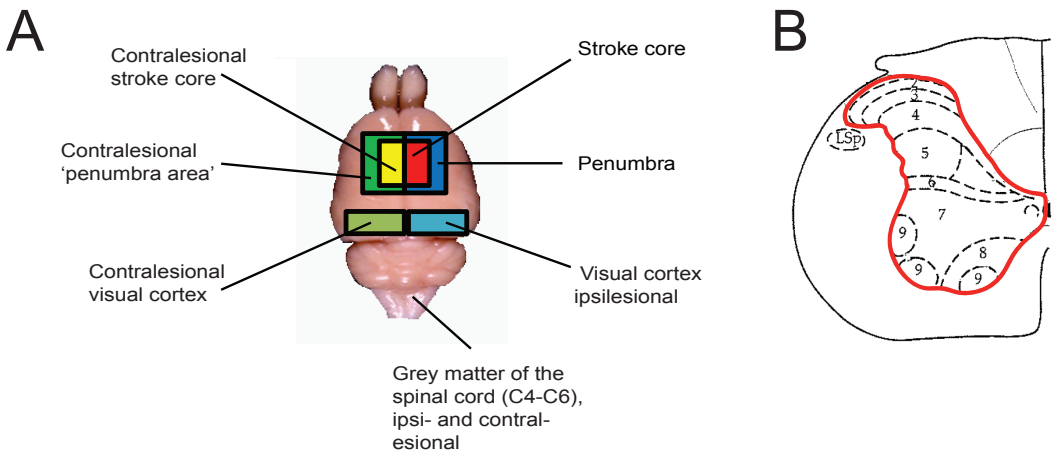

Fig. 1: Experimental set-up to analyze spatio-temporal expression patterns of growth promoting genes in the brain and spinal cord. (A) Schematic illustration depicting the tissue dissection for RNA extraction from the stroke core, the penumbra, the spinal cord and the visual cortex and from the corresponding areas in the contralesional hemisphere. (B) Schema showing grey matter extraction from the cervical hemi spinal cord to analyze modifications in gene induction in the ispsi- and contralesional spinal cord.

(*GAPDH*) (forward, 5'-CAGCAATGCATCCTGCACC-3'; reverse, 5'-TGGACTGTGGTCATG AGCCC-3'), growth associated protein 43 (*GAP43*)(forward, 5'-GGAAGATACCATGCTGT-3'; reverse, 5'-TATGAGCCTTATCCTCCGGT-3'), Brain-derived neurotrophic factor (*BDNF*), (forward, 5'-CAAAGCCACAATGTTCCACCAG-3'; reverse, 5'-AGTTGCCTTGTCCGTGGA CGTT-3'), Cyclic AMP-dependent transcription factor (*ATF-3*), (forward, 5'- CCTCTGGAG TGTCAGTCAC-3'; reverse, 5'-GCAGGCACTCTGTCTTCTCT-3'), fibroblast growth factor (*FGF2*) (forward, 5'- GGCTGCTGGCTTCTAAGTGT-3'; reverse, 5'-TCCGTGACCGGTAA-GTGTTG-3'), Synapsin Ib (*SYN*) (forward, 5'- CAAGAAGCTTGGAACAGAGG-3'; reverse, 5'- CCTGGAAGTCATGTTGGTTG-3'), Glial fibrillary acidic protein (*GFAP*) (forward, 5'-AGTGGTATCGGTCCAAGTTTGC-3'; reverse, 5'-TGGCGGCGATAGTCATTAGC-3'), Signal transducer and activator of transcription 3 (*STAT3*) (forward, 5'-GGCCCTTAGTCATCAA GACTGGTG-3'; reverse, 5'- AGGAAATTTGACCAGCAACCTGAC-3'), GAIP-interacting protein, C terminus (*GIPC*) (forward, 5'- TCGAGGGCTTCACTAATGTCAAGG-3'; reverse, 5'- TGAGGGTACAGAACATCACCTCAG-3').

Statistical analysis

The statistical analysis was performed with GraphPad Software, Prism 5. Bar graphs are presented as mean ±SEM. Statistical analyses were performed by using a two-way ANOVA followed by a post-hoc Bonferroni test. The correlation of qPCR data and behavioral performance of animals, Pearson correlation was applied. The level of significance was set at $^{*}P < 0.05, ^{**}P < 0.01$, $^{***}P < 0.001$.

5.3 Results

5.3.1 Stroke induces a distinct spatial and temporal expression profile for growth- associated genes and neurotrophic factors.

We performed a literature based screening for growth-associated genes and neurotrophic factors which were described to be up-regulated after stroke ($>$ 5 x-fold changes) and have been correlated with plasticity and/or functional recovery (Kinouchi et al., 1994; Akins et al., 1996; Soriano et al., 2000; Kim et al., 2002; Lu et al., 2003; Mitsios et al., 2007; Büttner et al., 2008; Li et al., 2010; Shono et al., 2010). We chose candidate genes which were associated with distinct growth-promoting functions within a specific context. In an early response to ischemia (Mattson, 2008; Carmichael, 2012), several neurotrophic factors such as brain-derived neurotrophic factor (*BDNF*), nerve growth factor (*NGF*) and neurotrophin3 (*NT-3*) and fibroblast growth factor (*FGF-2*) are induced. While *BDNF*, *NGF*, *NT-3* and *FGF-2* are involved in neuronal survival, neurogenesis, synaptogenesis and angiogenesis (Maisonpierre et al., 1990; Acheson et al., 1995; Huang and Reichardt, 2001; Freeman et al., 2004), *BDNF* in particular is known for its crucial role in learning and memory processes (Yamada and Nabeschima, 2003) as well as its association to accelerated sensorimotor recovery after stroke (Schabitz et al., 2004; 2007). Another candidates for our screen were the Growth Associated Protein 43 (*GAP43*), Synaptophysin I *(SYN)*, Glial fibrillary acidic protein (*GFAP*) and the Cyclic AMP-dependent transcription factor 3 (*ATF-3*).*GAP43* is expressed in neuronal growth cones during development and during axonal regeneration (Benowith and Routtenberg, 1987;). Synaptophysin I *(SYN)* as a synaptic marker is up-regulated by exercise in the naive and impaired animal (Vaynman et al., 2004; Ploughman et al., 2007). The Glial fibrillary acidic protein (*GFAP*) gene is usually expressed in astrocyte cells (Jaques et al., 1978) and has been also shown to play an important role in neuronal repair (Onose et al., 2009). The transcription factor *ATF3* was chosen as its neuroprotective role has been depicted after ischemic brain damage (Zhang et al., 2011; Ahlgren et al., 2011). We examined gene expression levels using qRT-PCR in the stroke core, the penumbra and the ispilateral visual cortex at 7 and 28 days after a large photothrombotic stroke in the sensorimotor cortex. We also were interested in modifications of growth promoting and neuroprotective genes in the corresponsing areas of the unimpaired contralateral hemisphere (Figure 1). We found that *BDNF* was significantly induced in the penumbra region (a frame of 2.5mm around the stroke core) at both time points (two-way repeated measures ANOVA with posthoc Bonferroni, $^{**}P < 0.01$, Fig. 2A). *FGF2* mRNA levels were increased >13 x-fold and for *ATF3* mRNA >169 x-fold increase was found in the stroke core 28 days after injury compared to naive control samples from the same region (Fig. 2C, D). Expression of *GFAP* mRNA was significantly enhanced in the penumbra and the corresponding contralesional sensorimotor cortex 7 days post insult (two-way repeated measures ANOVA with posthoc Bonferroni, $^{**}P < 0.01$, $^{***}P < 0.001$, Fig. 2E).

5.3.2 Expression patterns of growth promoting genes in the brain differ among different rehabilitation paradigms

In chapter 4 we have described that timing matters when rehabilitative training is applied in combination with Anti-Nogo immunotherapy after stroke. We found that animals which first received two weeks of Anti-Nogo antibody followed by two weeks of intensive training ($'Anti-Nogo-A/sequential'$) in the single pellet grasping task showed an almost complete recovery at the end of the rehabilitation schedule (Chapter 4, Fig. 1A, B). In contrast, concurrent training together with Anti-Nogo A immunotherapy ($'Anti-Nogo-A/parallel'$) resulted in the worst outcome with a recovery rate <12%. Animals in the control group which received

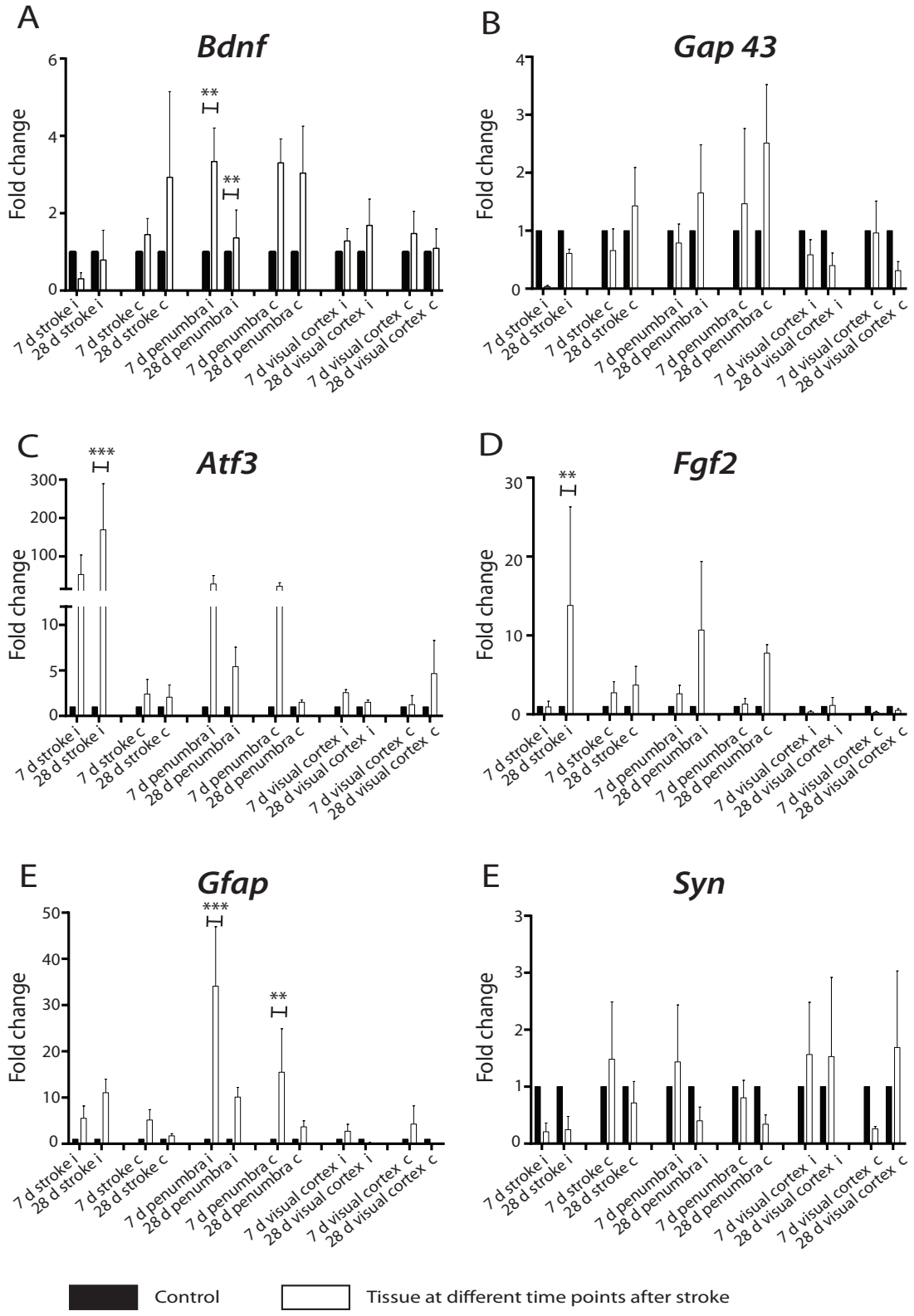

Fig.2: Gene expression analysis of growth associated proteins and neurotrophic factors. mRNA levels were measured by qRT-PCR from tissue taken at the stroke core ('stroke i'), or the contralesional corresponding sensorimotor cortex ('stroke c'), the penumbra ('penumbra i') or the corresponding area in the contralesional hemisphere ('penumbra c') as well as the ispi- ('visual cortex i') or contralesional visual cortex ('visual cortex c') 7 or 28 days after stroke surgery relative to control tissue from the same areas in naïve rats. Stroke induced a significant up-regulation of *BDNF* and *GFAP* in the penumbra at 7 days post stroke, while *ATF3* and *FGF2* mRNA levels were enhanced in the stroke core 28 days post insult (sample size n=3-4 per group and condition). Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-way ANOVA repeated measure followed by Bonferroni post hoc, asterisks indicate significances: $^{**}P < 0.01$, $^{***}P < 0.001$

early training ($'control/parallel'$) had a tendency to excell control animals which were trained after the application of control Ig G antibody ($'control/sequential'$). The distinct outcomes due to the different rehabilitation paradigms were reflected by different anatomical sprouting patterns of newly growing corticospinal fibers. We hypothesized that the different rehabilitation paradigms induced diverse intrinsic molecular expression patterns leading to the hardware changes we had found in our anatomical analysis (chapter 4, Fig. 2). We extracted RNA from tissue of the stroke core, penumbra and visual cortex as well as the corresponding contralesional areas (Fig. 1A) in all 4 rehabilitation groups 28 days post stroke as described above. The focus again lay on the analysis of enhanced mRNA expression of the same growth associated genes and neurotrophic factors.

Indeed, we found that distinct rehabilitation paradigms induce distinct patterns of neurotrophic factors and growth promoting genes in the brain. In particular the Anti-Nogo-A/sequential group, which depicted the highest level of functional recovery, also had the highest mRNA levels of the neurotrophic factors *BDNF* and *FGF2*, as well as the growth promoting gene *GAP43* at distinct locations relative to the stroke core (Fig. 3): *BDNF* induction was significantly higher in the stroke core and both visual cortex areas in the Anti-Nogo-A/sequential group than in all other rehabilitation groups, while *FGF2* and *GAP43* expression was significantly enhanced in the penumbra region of this group (two-way repeated measures ANOVA with posthoc Bonferroni, $^{*}P < 0.05$,$^{**}P < 0.01$, ***, Fig. 3A,B and D).

In the Anti-Nogo-A/parallel group we in particular detected high mRNA levels of *GFAP* as signs of gliosis and scar formation while *BDNF* levels in the same area were even slightly below controls (Fig. 3E). Significant *GFAP* induction was also found in the stroke core area of the 'control/parallel' group. Both groups, the $'Anti-Nogo-A/parallel'$ and the $'control/sequential'$ group, had demonstrated the poorest degree of forelimb restoration 4 weeks after stroke (Fig. 3E).

The anti-Nogo-A parallel group was also significant for enhanced expression of the synaptic marker Synapsin Ib in the penumbra and ipsilesional visual cortex (>57 x-fold change compared to naive control tissue in the same region, two-way repeated measures ANOVA with posthoc Bonferroni, $^{*}P < 0.05$,$^{**}P < 0.01$, Fig. 3F).

Stroke core tissue of animals in the $'control/parallel'$ which had received early intensive training after stroke induced the highest mRNA expression levels of the neuroprotective transcription factor *ATF3* compared to all other groups (two-way repeated measures ANOVA with posthoc Bonferroni, ***, Fig. 3C). This group performed second with a final recovery rate of $35-40\%$ after the $'Anti-Nogo-A/sequential'$ group (received a success rate of >85%) (Chapter 4, Fig. 1 A,B).

Expression patterns of growth promoting genes in the spinal cord differ among different rehabilitation paradigms

We also analyzed grey matter tissue of the lesioned and unlesioned cervical hemi spinal cord (at level C4 to C6) to detect differences in expression patterns of growth promoting genes in the four rehabilitation groups versus naive control tissue or animals without rehabilitation after stroke. We again found that the growth promoting genes *BDNF*, *GAP43* and *FGF2* were significantly more enhanced in the spinal cord for animals in the $'Anti-Nogo-A/sequential'$ group, the group with the best functional outcome, than in all other groups (two-way repeated measures ANOVA with posthoc Bonferroni, $^{*}P < 0.05$,$^{**}P < 0.01$, Fig. 4A, B, C).

Here we show in particular that the increased induction of mRNA levels of *BDNF* and the Signal transducer and activator of transcription 3 (*STAT3*) in the impaired hemi spinal cord were specific for the $'Anti-Nogo-A/sequential'$ group in this experimental set-up. *STAT3* is a protein which is activated through phosphorylation of tyrosine 705 (Yuan et al., 2004), in

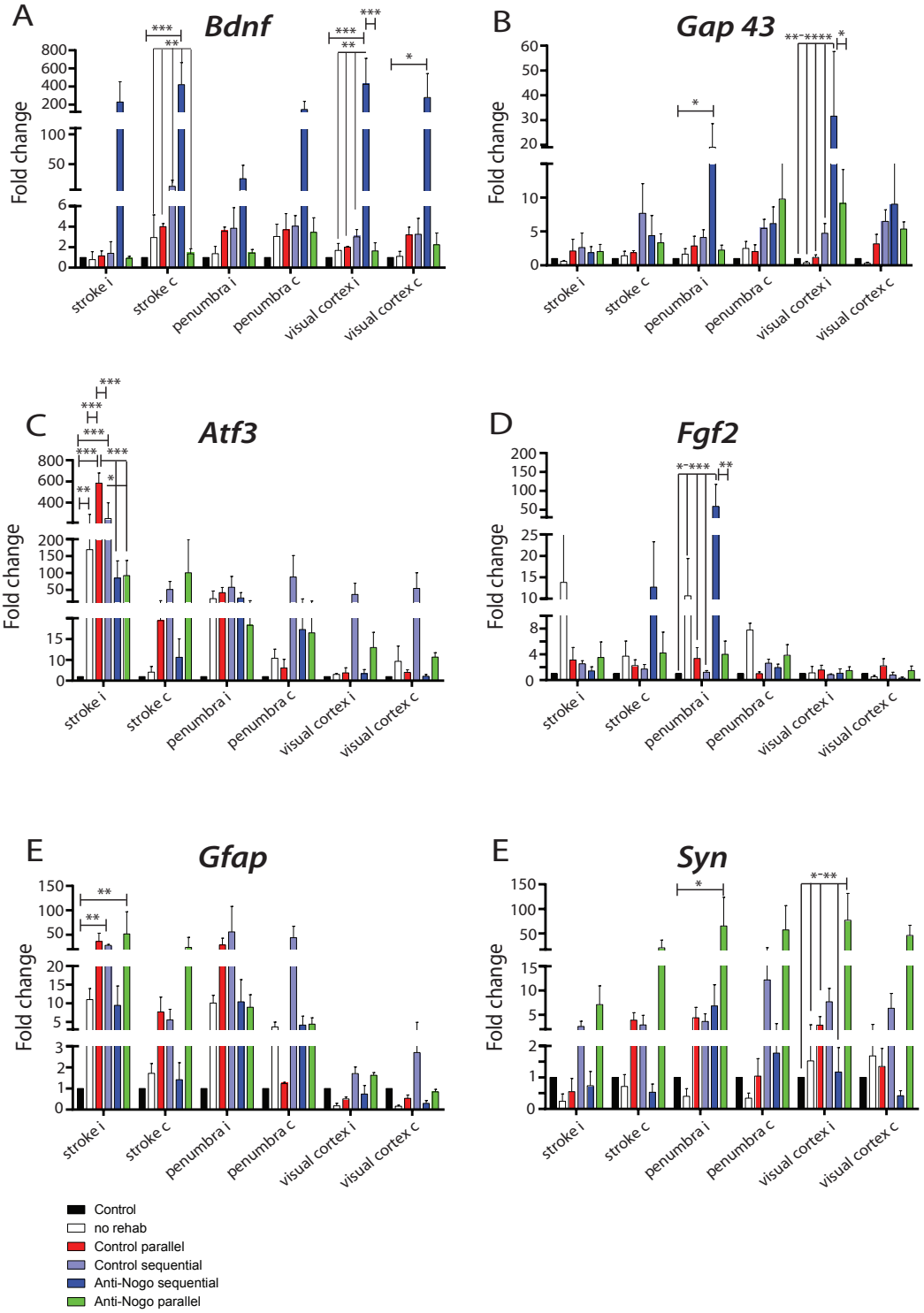

Fig. 3: Distinct rehabilitation schedules induce diverse spatio-temporal expression patterns of growth promoting genes and neurotrophic factors in the brain 28 days after stroke. mRNA levels were measured by qRT-PCR from tissue taken at the stroke core ('stroke i'), or the contralesional corresponding sensorimotor cortex ('stroke c'), the penumbra ('penumbra i') or the corresponding area in the contralesional hemisphere ('penumbra c') as well as the ispi- ('visual cortex i') or contralesional visual cortex ('visual cortex c') from rats without rehabilitation ('spontaneous recovery' group) or from rats of the four rehabilitation paradigms ('Control/parallel', 'Control/sequential', 'Anti-Nogo-A/sequential', 'Anti-Nogo-A/parallel') versus control tissue from the same areas in naïve rats (sample size n=3-4 per group and condition). mRNA levels of the growth associated genes *BDNF*, *Gap43* and *FGF2* were highly up-regulated in the Anti-Nogo-A sequential group (A, B, D), the group with the best functional recovery of skilled motor function (Chapter 4, Fig. 1). In contrast, the Anti-Nogo-A parallel group was significant for signs of increased astrocytosis (E) and enhanced expression of the synaptic marker synapsin Ib in the penumbra (F). Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-way ANOVA repeated measure followed by Bonferroni post hoc, asterisks indicate significances: $^{**}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

response to various cytokines and growth factors including interferons and interleukins as well as a downstream signaling factor for growth factors such as epidermal growth factor (*EGF*), the leukemia inhibitory factor (*LIF*) and the ciliary neurotrophic factor (*CNTF*)(Atreya s et al., 2006; Jablonka et al., 2014). *CNTF* signaling via the Jak/Stat-3 pathway has been described to play a major role in axonal regeneration of retinal ganglion cells (Park et al., 2003; Muller et al., 2007) and motoneurons (Jablinka et al., 2014). We found that changes for both genes, *STAT3* and *BDNF*, were significantly higher in $'Anti-Nogo-A/sequential'$ animal compared to the $'Anti-Nogo-A/parallel'$ group, which had displayed the poorest functional recovery rate (two-way repeated measures ANOVA with posthoc Bonferroni, $^{*}P < 0.05$, Fig. 4 A, G). In contrast, mRNA levels of synapsin Ib (*SYN*) were exclusively elevated in the contralesional hemi spinal cord in $'Anti-Nogo-A/parallel'$ animals compared to naive controls and all other rehabilitation groups (two-way repeated measures ANOVA with posthoc Bonferroni, $^{*}P < 0.05$,$^{**}P < 0.01$, Fig. 4F), reflecting increased synaptic turn-over which we have also detected on the anatomical level (Chapter 4, Fig. 2G). The $'Anti-Nogo-A/parallel'$ group was also significant for the highest x-fold changes for *GFAP* in the spinal cord (Fig. 4, E). We also found significantly increased mRNA levels of the gene coding for GAIP-interacting protein, C terminus (*GIPC*)exclusively expressed in the contralesional hemi spinal cord of the $'Anti-Nogo-A/parallel'$ group. *GIPC* is a regulatory protein in the postsynaptic density for extrasynaptic NMDA receptor trafficking (Yi et al., 2007) which is associated with NMDA receptor (NMDAR)-induced excitotoxicity contributing to cell death in certain neurodegenerative diseases, stroke, epilepsy, and traumatic brain injury (Hadingham and Bading, 2010; Parsons and Raymond, 2014).

5.3.3 Increased *BDNF* mRNA levels in the contralesional hemi spinal cord correlate with good functional outcome

As described in Chaper 4 we have found that animals which first received Anti-Nogo-A immunotherapy followed by 2 weeks of training showed the best recovery to nearly full forelimb function at the end of the rehabilitative training in the single pellet grasping task. We found in this group the highest expression patterns of *BDNF* mRNA levels, both in the brain (contralesional sensorimotor cortex ('stroke c', Fig. 3a) and ipsilateral visual cortex, BDNF x-fold induction >400-fold compared to naive control set to 1, Fig. 3A) and the spinal cord (impaired spinal hemi cord, BDNF x-fold induction >10-fold compared to naive control set to 1, Fig. 4A) while no significantly enhanced *BDNF* mRNA levels were detected in the other tested rehabilitation paradigms or the group with spontaneous recovery after stroke. In particular, *BDNF* mRNA levels in the contralateral healthy spinal hemi cord positively correlated with the final success scores of animals in the $'Anti-Nogo-A/sequential'$ group 4 weeks after stroke onset (r=0.93, Pearson's correlation, Fig. 5B). There was no correlation of *BDNF* gene expression levels with final success rates or recovery rates at the end of the rehabilitative training in any of the other rehabilitation groups or for animals with spontaneous recovery.

5.4 Discussion

BDNF as a prognostic marker for functional outcome levels after stroke?

Several studies have described how rehabilitative training and enriched environment rehabilitation influence not only outcome levels for motor recovery but also modify temporal profiles of growth factors after cerebral ischemia (Vaynman and Gomez-Pinilla, 2005; Ploughman et al.,

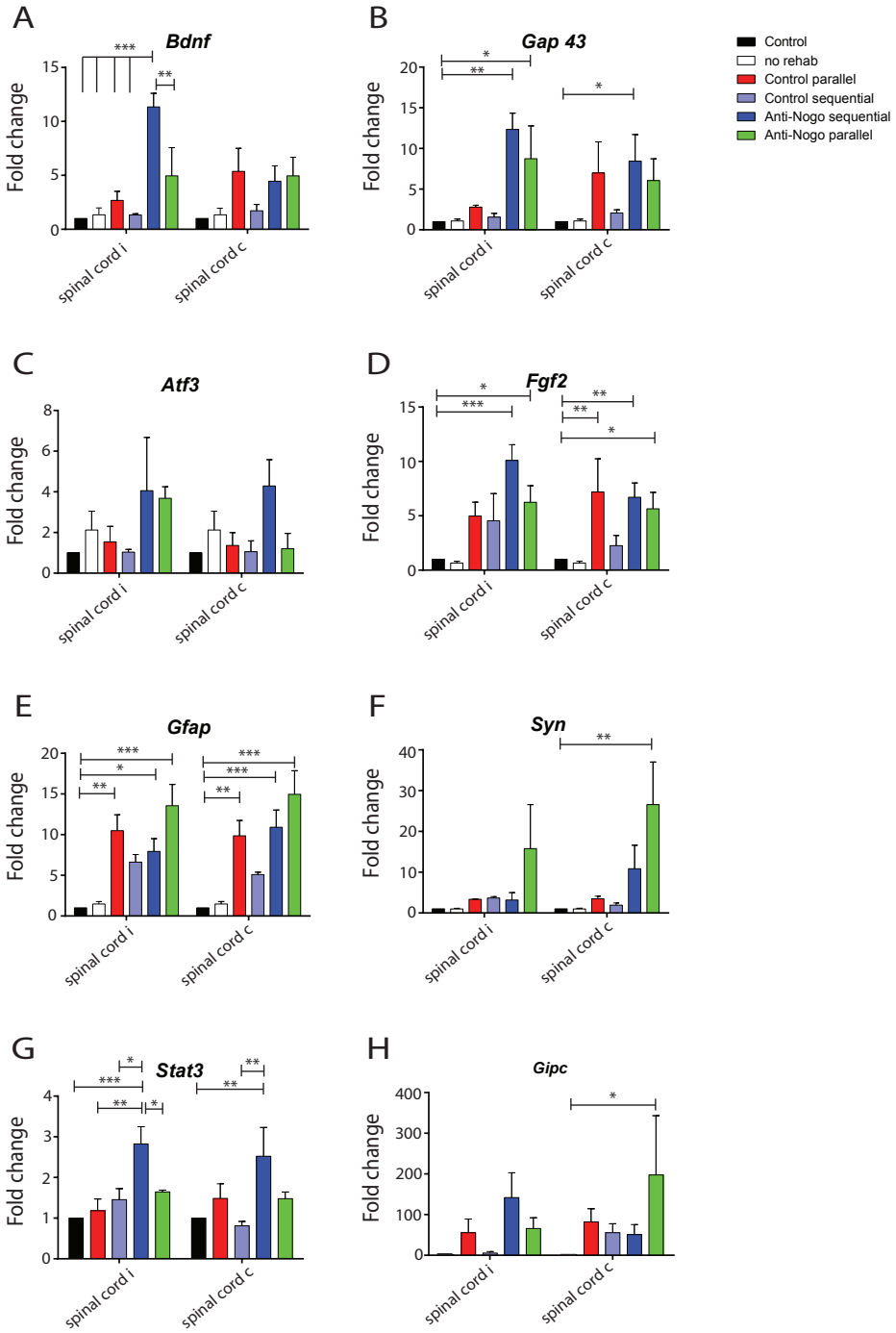

Fig. 4. Timing of rehabilitative paradigms differently influences expression of growth associated genes in the cervical spinal cord 28 days after stroke. mRNA levels were measured by qRT-PCR in tissue from the impaired ('spinal cord i') or contralateral healthy cervical hemi spinal cord ('spinal cord c') at level C4-C6 from rats without rehabilitation ('spontaneous recovery' group) or from rats of the four rehabilitation paradigms ('Control/parallel', 'Control/sequential', 'Anti-Nogo-A/sequential', 'Anti-Nogo-A/parallel') versus control tissue from the same areas in naïve rats (sample size n=3-4 per group and condition). (A) *BDNF* expression levels were significantly higher up-regulated in the 'Anti-Nogo-A/sequential' group than in all other rehabilitation groups in the de-nervated hemi spinal cord. (B, D) Both rehabilitation groups treated with Anti-Nogo antibody ('Anti-Nogo-A/sequential', 'Anti-Nogo-A/parallel') revealed increased levels of *GAP43* and *FGF2* in the de-nervated hemi spinal cord. (C) In non of the experimental groups a significant modification of *ATF3* gene expression was detected. The 'Anti-Nogo-A/parallel' group depicted the highest mRNA levels for *GFAP* among all rehabilitation groups in the spinal cord (E), but was the only group with significant up-regulation of the synaptic marker synapsin Ib (F). *STAT3*, as part of the growth promoting Jak/STAT3 pathway was exclusively enhanced in the 'Anti-Nogo-A/sequential' group (G) while GIPC expression pattern in the 'Anti-Nogo-A/parallel', a protein associated with extrasynpatic NMDA receptor signaling for cell death promoting, excelled mRNA levels in all other rehabilitation groups (H). Data are presented as means ± s.e.m.; statistical evaluation was carried out with two-way ANOVA repeated measure followed by Bonferroni post hoc, asterisks indicate significance: $^{**}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

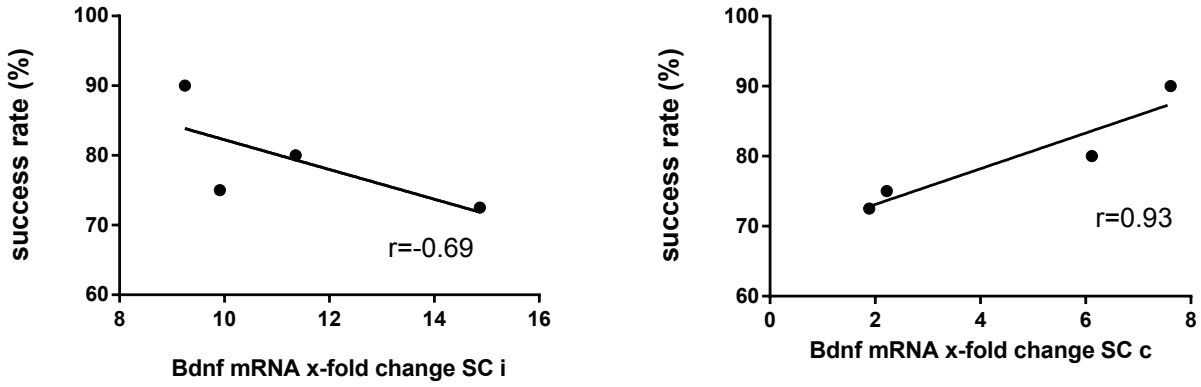

Fig. 5. *BDNF* expression in the spinal cord correlates with regained fine motor skills in the single pellet grasping task 28 days after stroke. Success rates 28 days post stroke, at the end of the rehabilitative schedule from animals which first received 2 weeks of Anti-Nogo-A immunotherapy followed by 2 weeks of intense rehabilitative training in the single pellet grasping task ('Anti-Nogo-A/sequential' group) were compared with *BDNF* mRNA levels in the impaired ('SC i') and contralateral healthy ('SC c') cervical hemi spinal cord. Only *BDNF* mRNA levels in the healthy, contralateral hemi spinal cord correlated to the final success rates (B, r=0.93, Pearson' s correlation) while there was no correlation between success rates in the single pellet grasping task and BDNF expression in the de-nervated ('i'= impaired) hemi spinal cord (A, r=-0.69, Pearson' s correlation).

2007; Gelfo et al., 2011; MacLellan et al., 2011; Zeiler and Krakauer, 2013). Here we show that applying rehabilitative training concurrently ($'Anti-Nogo-A/parallel'$ group) or following Anti-Nogo-A immunotherapy ($'Anti-Nogo-A/sequential'$ group) resulted not only in two distinct outcome levels (10% final success rate for the $'Anti-Nogo-A/parallel'$ group versus >86% success rate for the $'Anti-Nogo-A/sequential'$ group at the end of the rehabilitative scheduel, 4 weeks after stroke surgery, chapter 4), but also revealed two distinct expression patterns of growth promoting genes and neurotrophic factors. In particular brain and spinal cord tissue of animals in the $'Anti-Nogo-A/sequential'$ group was significant for increased mRNA levels of *BDNF*, *Gap43* and *FGF2* (Fig. 3, 4). However, these genes were enhanced at specific areas in relation to the stroke core 4 weeks post stroke: While *Gap43* and *FGF2* mRNA levels were up-regulated in the penumbra region of the stroke (Fig. 3B, D), expression of *BDNF* was increased in remote areas to the stroke core- such as the contralesional sensorimotor cortex ('stroke c') and the ispi- and contralateral visual cortex (Fig. 3A).

BDNF along with the insulin-like growth factor (*IGF-1*) and the vascular endothelial-derived growth factor (*VEGF*) is one of the principal growth factors known to mediate the effects of exercise on the brain (Cotman etal., 2007). Up-regulation of *BDNF* in the brain and spinal cord post CNS injury have been reported repeatedly (Mocchetti and Wrathall, 2005; Vaynman et al., 2005). In particular, increased recovery rates after rehabilitative training or enriched rehabilitation was associated with enhanced levels of *BDNF* expression (Vaynman et al., 2006; Kazlaukas et al., 2011; Gelfo et al., 2011; MacLellan et al., 2011). Administrating BDNF improved sensorimotor recovery in rats after ischemia (Schabitz et al., 2004; Muller et al., 2008), whereas blocking endogeneous *BDNF* with antisense oligonucleotide (Ploughman et al., 2009) or blockade of *TrkB*, the BDNF receptor, (Vaynman et al., 2004; Vaynman et al., 2006) with antibodies attenuated the positive effects of rehabilitative exercise and learning. The current data also support the role of *BDNF* as a marker for enhanced recovery levels due to a specific rehabilitation paradigm after stroke: *BDNF* mRNA levels in the contralesional cervical hemi spinal cord correlated with final success rates in the $'Anti-Nogo-A/sequential'$ group (Fig. 5), while no induction and no correlation was detected for the $'Anti-Nogo-A/parallel'$ rehabilitation

group depicting the poorest outcome. A similar effect was described by Griesbach et al., 2004a: In a traumatic brain injury model premature exercise had inhibited *BDNF* up-regulation and resulted in impaired recovery of cognitive function and even precluded the normal induction of plasticity molecules regulated by *BDNF* action, such as *CREB* and synapsin I (*SYN*). However, delayed exercise 14 days post injury enhanced *BDNF* levels and cognitive function (Griesbach et al., 2004b).

Do enhanced BDNF levels indicate a prolonged plastic phase after stroke?

The wave-like precisely timed induction of growth-promoting and inhibiting genes (Carmichael et al., 2005) after stroke as well as recent behavioral studies in humans and animals indicate a limited period of heightened neuroplasticity, comparable to that what occurs during visual system development (Murphy and Corbett et al., 2009). In one first important experiment only early exposure to enriched rehabilitation (5 or 14 days post stroke) resulted in significant recovery in a rat stroke model, whereas rats given delayed training (30 days post stroke) exhibited little improvement (Biernaskie et al., 2004). In the current study the sequential application of first Anti-Nogo-A immunotherapy for two weeks followed by 2 weeks of intense rehabilitative training of the impaired forelimb had not only resulted in the best functional outcome compared to all other tested rehabilitation groups but also in the induction of increased *BDNF* levels in the brain and spinal cord in these animals. The application of Anti-Nogo-A immunotherapy may have expanded the time window for increased plasticity. The consecutive training may not have only guided processes for specific circuit selection and stabilization as well as pruning of unspecific neuronal rewiring, but may also have maintained areas plastic remote from the stroke core through exercise and by the induction of *BDNF*.
In contrast, as we just analyzed gene expression patterns after the completion of all rehabilitative schedules 4 weeks after stroke surgery, we cannot rule out increased *BDNF* mRNA levels in the $'Anti-Nogo-A/parallel'$ group earlier after stroke, presumably 14 days post insult, when the training concurrently to the Anti-Nogo-A immunotherapy had been completed. Enhanced *BDNF* levels at that time may have induced *synapsin I* mRNA which we detected to be exclusively up-regulated ($>$ 20 x-fold compared to naive controls in the spinal cord, Fig. 4F) for this groups 4 weeks after stroke. The ability of *BDNF* to regulate components of the synaptic release machinery such as *synpasin I* under exercise conditions has been described elsewhere (Molteni et al., 2002; Vaynman et al., 2003). Interestingly, synapsin I also regulates neuronal developmental processes, e.g. the formation and maintenance of presyaptic structures, axonal elongation and new synaptic formation (Ferreira et al., 1998). An early extensive up-regulation of *BDNF* by simultaneous training and Anti-Nogo-A antibody application in the $'Anti-Nogo-A/parallel'$ group may have exaggerated BDNF-levels and consecutively initiated excessive expression of synapse formation and regulation proteins such as *synapsin I*. Thus, this may have created the aberrant and limitless neuronal sprouting and synaptogenesis detected in the anatomical analysis of spinal cord slices for this group (Chapter 4, Fig. 2).

The *STAT3*-pathway is induced in the $'Anti-Nogo-A/sequential'$ group

In recent years the activation of the *Jak/Stat3* pathway has been identified as one of the central players that regulate intrinsic growth programs (Aaronson and Horvath, 2002). *STAT3* expression induces neuronal outgrowth in vitro (Smith et al., 2011) and enhanced *STAT3* expression associated to the conditioning response of DRG neurons (Pernet et al., 2013). In addition, increased levels of *STAT3* expression and phosphorylation are involved in axonal regeneration

(Schwaiger et al., 2000; Sheu et al., 2000) and axonal remodeling (Bareyre et al., 2002). The role of *STAT3* in axonal regeneration has been in particular described as an initiator for precisely timed initiation of axonal regeneration in the peripheral nervous system but does not participate in the subsequent elongation of PNS axons (Bareyre et al., 2011). *STAT3* was up-regulated in sprouting unlesioned fibers from the contralateral, right forelimb portion of the corticospinal tract in response to the unilateral denervation by pyramidotomy in a rat spinal injury model (Lang et al., 2013). These newly formed collaterals extended towards the denervated side of the spinal cord resulting in a significant increase in the number of midline crossing fibers (Lang et al., 2013).
In the current study *STAT3* mRNA levels were in particular significantly up-regulated in the $'Anti-Nogo-A/sequential'$ group in both, the ipsi- and the contralesional hemi spinal cord (Fig. 4G) while there was no significant enhancement of *STAT3* expression in any of the other rehabilitation groups. The $'Anti-Nogo-A/sequential'$ group was also the group with the highest number of midline crossing corticospinal fibers growing from the intact hemi spinal cord across the midline of the central canal to the denervated side (Chapter 4, Fig. 2). We hypothesize that the *STAT3* pathway was involved in this recruitment of unlesioned corticospinal fibers whereas the enhancement of ipsilateral projecting corticospinal fibers maintained and exceeded the number of recruited unlesioned contralateral corticospinal fibers for circuit remodeling in animals of the $'Anti-Nogo-A/parallel'$ group. Nevertheless, whether the *STAT3* pathway was induced by molecules such as the neuropoietic cytokines *IL-6*, ciliary neurotrophic factor, and leukemia inhibitory factor, as well as the intracellular regulator *SOCS3*, which have been shown to influence axonal regeneration (Cafferty et al., 2001, 2004; Smith et al., 2009), has to be elucidated.

Tissue of the $'Anti-Nogo-A/parallel'$ group reveals signs of enhanced astrocytosis

After stroke, reactive astrocytes proliferate, migrate and form a glial scar around the injured brain tissue (Ostergaard and Jensen, 2013). However, the role played by astrocytosis for the functional recovery remains ambiguous: Although positive features of reactive astrocytosis exist - such as the establishment of structural support for cellular elements, the release of growth factors, the maintenance of the blood-brain-barrier and the reestablishment of extracellular homeostasis (Pekny and Nilsson, 2005), most authors associate increased astrocyctic markers with negative aspects: The inhibition of axonal growth through the formation of a physical barrier (glia scar) as well as the secretion of factors inhibitory to axon growth cones may negatively influence recovery levels after brain injury (Abeysinghe et al., 2014). We found enhanced *GFAP* expression in the stroke core of the$'Anti-Nogo-A/parallel'$ group and the $'Control/sequential'$ group, (Fig. 3E). This indicates strong glial scar formation in the stroke core which may have aggravated remodeling and reorganization processes thus contributing to the poor functional outcome seen in those two rehabilitation groups.
The contralesional hemi spinal cord of animals in the $'Anti-Nogo-A/parallel'$ group was also significant for increased mRNA levels of *GIPC* (Fig. 4H). The PDZ protein GIPC is preferentially associated with extrasynaptic NMDA receptors and may play a role in their organization and trafficking (Yi et al., 2007). Increased extrasynaptic NMDA receptor signaling has been described to promote cell death through excitotoxic events (Hardingham and Bading, 2010). However, if excitotoxic events with consecutively exaggerated neuronal apoptosis contributed to the poor recovery level of animals in the $'Anti-Nogo-A/parallel'$ group remains to be enlightened.

Conclusion and Outlook

This study described that distinct rehabilitative schedules induce specific spatio-temporal expression patterns of several growth promoting genes and neurotrophins. We identified factors such as *BDNF* and *STAT3* exclusively enhanced in tissue of animals with a particularly high functional recovery rate ($'Anti-Nogo-A/sequential'$ group) while signs of gliosis were found in the rehabilitation groups with less restoration of skilled forelimb function ($'Anti-Nogo-A/parallel'$ and $'Control/sequential'$ group). However the current study was conducted on a candidate based screening while deep sequencing or chip analysis of laser microdissected tissue may elucidate new molecular components playing a role in the intrinsic repair machinery of the central nervous system after stroke. Furthermore new state-of the art technology such as molecular profiling of neurons, interneurons and glia based on activity (Knight et al., 2012) and connectivity (Ekstrand et al., 2014) opens up possibilities to not only understand how extrinsic rehabilitative strategies influence internal repair mechanisms but also to develop new therapeutic concepts to maximize repair of lost functions for stroke patients.

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Part III

Conclusion and Outlook

This thesis aims at understanding basic principles of stroke-induced cortical reorganization as well as analyzing the role of neurorehabilitation on plastic mechanisms after ischemic insult.
From both, clinical and experimental studies, it is well known that the capacity of the brain and spinal cord to induce functional recovery highly depends on the size of the stroke and its location.
In a first step we studied the reorganization in the peri-infarct area after a small focal stroke destroying the forelimb motor cortex in Thy1-ChR2 transgenic mice. After an initial decline of skilled forelimb function due to the stroke, animals fully recovered without any further intervention within 4 weeks after stroke surgery. This regain of function was accompanied by a map shift of the forelimb area towards the hindlimb motor cortex as measured by longitudinal light-based motor mapping within the same animal over the course of weeks post stroke. In addition, the cortical representation of the hindlimb expanded diffusely and stimulus-evoked responses were increased indicating a lowered threshold for corticospinal signal transduction. Detailed analysis of grasping kinematics revealed two stereotypic error patterns which appeared within the first week after stroke onset: Animals grasped too long and therefore failed to target the rung or targeted the rung with the wrist and consecutively slipped due to missed grasping force of the digit and MCP joint around the rung. We then used a pharmacogenetic approach to temporarily and reversibly silence neurons in the peri-infarct area in order to test the functional relevance of the penumbra area for the regain of skilled forelimb function. And indeed, 30 min after CNO application, animals showed a decline of the fully regained forelimb function, comparable to the decrease of success rates in the two grasping tasks assessed within the first week after stroke. As detailed analysis of grasping kinematics revealed that the same grasping errors stereotypical for the grasping pattern 1-3 days after stroke reemerged 30 min after CNO application, a first indirect proof was delivered that the motor engram formed during the pre-stroke training was re-established within the newly formed center for forelimb function after 4 weeks of stroke induced cortical reorganization. However there are limitations to this study. Analyzing single neuron activity in networks before, during and after stroke may elucidate not only the existence of specific microcircuits coding for specific movement parameters, but will furthermore allow to feature to which degree neurons are tunable to 'take over' a multitude of functions.

In our second study we combined two therapeutic treatment options to examine how different rehabilitative schedules influence functional outcome of forelimb motor skills in a rat stroke model. We choose 4 different rehabilitation paradigms: Intensive rehabilitative training of the forelimb was either combined concurrently to Anti-Nogo A immunotherapy immediately after stroke, or Anti-Nogo A immunotherapy was applied for 2 weeks after stroke followed by another two weeks of rehabilitative training. In accordance with this, control groups either received training simultaneously to Ig G control antibodies are afterwards (early versus delayed training). We found a very diverse picture of outcome levels depending on the rehabilitative paradigm we applied: Rats which first received Anti-Nogo A immunotherapy for two weeks followed by two weeks of rehabilitative training showed nearly full recovery of forelimb function (>86% recovery rate compared to baseline levels). In contrast, simultaneous application of Anti-Nogo A immunotherapy and rehabilitative training resulted in poor outcome levels (10% of baseline function) which were even lower than comparable levels of animals in the control groups or no rehabilitation at all.
Anatomical studies where we visualized the fibers coming from the motor forebrain cortex to the spinal cord revealed large numbers of fibers growing from the intact side across the midline in the cervical spinal cord in the animals treated with the anti-Nogo A antibody. In the well recovered group of rats, these fibers formed a near normal branching and termination pattern. In contrast, rats with early rehabilitation concurrently to the immunotherapy and poor func-

tional recovery showed overshooting fiber growth, aberrant branching and too high numbers of synapses, in part in wrong areas of the spinal cord. We then used two virus-based pharmacogenetic approaches for reversible short-and long-term inactivation of corticospinal fibers originating in the intact contralesional hemisphere which side-switched in the cervical spinal cord forming midline crossing fibers from the healthy spinal hemi cord to the denervated one. Silencing these newly outsprouting corticospinal fibers resulted in a decline of regained skilled forelimb function in animals, which had received Anti-Nogo-A immunotherapy followed by rehabilitative training before, indicating the functional relevance of these corticospinal fibers for the restoration of forelimb function. As the constructs for silencing the fibers were tagged with mCherry, we could identify the neurons sending these corticospinal midline crossing fibers. They were localized in a distinct area of the contralesional pre-motor and rostral primary motor cortex. We had shown for the first time, that the new formation of a distinct neuronal circuit was crucial for the re-establishment of lost motor function due to stroke. No compensation by other circuitry was apparent during the inhibition of these distinct subsets of midline crossing corticospinal fibers.
In the last part of the present work we aimed at gaining first insights into underlying molecular mechanisms for anatomical rewiring and functional recovery when distinct rehabilitative schedules are applied. We used a candidate-based approach to analyze if different rehabilitative paradigms induce distinct spatio-temporal expression patterns of growth-promoting genes and neurotrophic factors after stroke. We found that induction levels of BDNF - in particular in the unimpaired spinal hemicord were correlated with good functional outcome. Only spinal cord tissue of the group with excellent functional outcome (which had first received Anti-Nogo A immunotherapy followed by rehabilitative training) was significant for increased levels of *STAT3* - a pathway associated with enhanced axonal regeneration after CNS injury. In contrast, signs of gliosis (increased mRNA levels of *GFAP*) and excessive synaptogenesis (increased mRNA levels of *Synapsin Ib*) were characteristic for the rehabilitation group with the poorest level of performance at the end of the rehabilitative schedule - animals which had been trained concurrently to Anti-Nogo-A immunotherapy.

Our study shows the importance of carefully designing rehabilitation schedules after stroke, in particular if different therapeutic options such as training and growth enhancing agents are combined: A rehabilitative approach that supports the intrinsic potential of the brain and spinal cord to reinforce lost functions by boosting the growth of new fibers and by stabilizing the meaningful newly formed circuits may result in robustly enhanced recovery of motor function. In contrast, rehabilitative schedules may lose their beneficial character if applied at the wrong time and intensity. During an early phase after the injury, the brain seems to be in a particularly vulnerable state, and forced rehab therapies should therefore be applied with great caution during this period of circuit plasticity. These results have crucial implications also for designing clinical rehabilitation trials: By adding different drugs or training programs whereby each of them itself leads to a certain degree of functional improvement, you may not end up in added percentages of functional outcome levels and thus overall enhancement of function, but rather the opposite. Treatments may interfere with each other and with intrinsic plastic processes. Maybe, in a certain phase, the brain has to be left alone to do first its job and activate its own repair mechanisms. It needs first to be settled and prepare itself for external intervention. We may help the brain getting settled and prepared, meaning we need to find answers to a quantity of questions experimentally, till we face the challenge of a clinical setting.

Thus, the work presented here can just be a starting point. We have identified so far a rehabilitative design which provides excellent recovery of motor function in a rat model. We have analyzed the reorganization of a specific tract and by temporarily silencing it, found sup-

ported evidence that the newly outsprouting fibers of this tract are crucial for the restoration of skilled motor function. And we gained first hints that growth factor distribution is different among animals from different rehabilitation paradigms. However, according to the philosopher of science, Karl Popper, 'Science may be described as the art of systematic over-simplification. And specialization may be a great temptation for the scientist. For the philosopher it is the mortal sin.' How much of what we saw is true recovery and how much of it was compensation? Does in a simplified model rehabilitative training activate another pathway than Anti-Nogo A immunotherapy and are these pathways able to inactivate each other? What about sensory-feedback to shape the level of functional recovery? Thus, we have just opened the door to understand how molecular key players lay the basis for neuronal rewiring leading to a meaningful action and in the case of stroke to the restoration of lost function.
What kind of questions would we need to address next? When neurons within a network are destroyed by a stroke, we would like to know, which kind of other neurons take over, which kind of cell types? Do they just shoulder new function because they are spatially close to the destroyed ones or because they had a related function before? Are they tunable to a multi-set of functions? What is the molecular signaling behind the selection of one cell against another? If cells are selected – how do they find other selected cells? Which kind of signals do they send out, pure signs of electrical activity? How about chemo-attractant and chemo-repulsive molecular signals? Do cells keep other cells they are connected with active and thus alive by increased synaptic input? Are intact cellular networks - meaning cells which already wire and fire together, recruited after stroke to reorganize and establish function or are completely new cellular ensembles formed? And last but not least, how would the ideal experiment look like to examine all the questions raised?

In the ideal situation we would see how the stroke occurs, how first the neurons in the stroke core die. We could measure how other neurons are affected by the death-signal of the dying neurons and by inflammatory signals. We could see how the input to neurons connected to the dying cells is decreasing. We could monitor how they decide to degenerate or instead strengthen connections from which they get input- or, they even form new connections as a survival strategy.
How can we study all these events? A selective labeling of neurons directly affected by the stroke would be required as well as a labeling of their connections. We also would need specific labeling of newly outsprouting axons as well as markers for connections which get strengthened versus those that got lost. In addition, all labeling approaches can be combined with genetic profiling allowing the identification of underlying genomics and proteomics. Longitudinal studies in the same animal over time would be also essential to understand if connection strengthenings are robust and constant over time. Then ultimately, we could challenge the pathophysiological events by applying therapeutical interventions. In the ideal experiment, we study both, the network level as well as the molecular level while we analyze the behavioral output and determine if an intervention is beneficial or harmful. We zoom in from the behavior to the cellular and to the molecular event. All these means huge data accumulation and analysis. It also means a team effort including biologists, physicists, computer scientists, engineers and clinical neurologists to finally bridge the gap from experimental modeling to patient care. As Francis Crick in one of his Kuffler lectures at the University of California at San Diego in 1999 said, when he gave advice to neuroscientists to go to their molecular biologist colleagues to ask for appropriate techniques and new tools, 'we should discuss and tell what to do next. Once the word gets around that a certain type of problem exists it is surprising how often someone has a bright idea of how to solve it. So, don' t be shy - ask! After all, exactly how our brain works, is of vital interest to us all, so why shilly-shally.'

Part IV

Appendix

Curriculum vitae

Name	Dr. med. Anna-Sophia Elsa Wahl
Date of birth	March 20, 1985
Place of birth	Freudenstadt
Nationality	German

Education

Since July 2011	PhD Student in the lab of Prof. Martin E. Schwab, Brain Research Institute, ETH and UZH, Zurich, Switzerland
Since July 2011	Participant of the MD/PhD Programm, University of Zurich, Switzerland
2012	American Medical State Examination USMLE Step II
2011	Second Medical State Examination
October 2004-May 2011	Studies of medicine and biology at the University of Heidelberg, Germany
2007-2011	Participant of the MD/PhD Programm, University of Heidelberg, Germany
April 2007-April 2009	MD thesis "Pattern expression of pro-apoptotic genes in neurons: Examination of the Clca1 gene under hypoxic/ ischemic conditions" at the Interdisciplinary Center of Neurosciences, Heidelberg; Supervisor: Prof. Dr. H. Bading, Director; Co-Supervisor: Prof. Dr. M.Schwaninger Promotion date May 24, 2011
2006	First Medical State Examination
1995-2004	Kepler-Gymnasium Freudenstadt, June 2004: final examination (Abitur)
1991-1995	Primary school, Freudenstadt-Dietersweiler

Teaching Experience

Since July 2011	Teaching ETH students (lectures, practical courses etc.) Supervision of 2 Master students, Training of other PhD candidates

Awards

Scholarship of the German National Academic Foundation (Studienstiftung des Deutschen Volkes)

Scholarship of the Konrad-Adenauer Foundation e.V.

Publications

Wahl AS, Omlor W, Rubio JC, Chen JL, Zheng H, Schröter A, Gullo M, Weinmann O, Kobayashi K, Helmchen F, Ommer B, Schwab ME. Neuronal repair. Asynchronous therapy restores motor control by rewiring of the rat corticospinal tract after stroke. Science. 2014 344(6189):1250-5. doi: 10.1126/science.1253050.

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S.D.G.

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Finding an optimal rehabilitation paradigm after stroke: enhancing fiber growth and training of the brain at the right moment

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After stroke the central nervous system reveals a spectrum of intrinsic capacities to react as a highly dynamic system which can change the properties of its circuits, form new contacts, erase others, and remap related cortical and spinal cord regions. This plasticity can lead to a surprising degree of spontaneous recovery. It includes the activation of neuronal molecular mechanisms of growth and of extrinsic growth promoting factors and guidance signals in the tissue. Rehabilitative training and pharmacological interventions may modify and boost these neuronal processes, but almost nothing is known on the optimal timing of the different processes and therapeutic interventions and on their detailed interactions. Finding optimal rehabilitation paradigms requires an optimal orchestration of the internal processes of re-organization and the therapeutic interventions in accordance with defined plastic time windows. In this review we summarize the mechanisms of spontaneous plasticity after stroke and experimental interventions to enhance growth and plasticity, with an emphasis on anti-Nogo-A immunotherapy. We highlight critical time windows of growth and of rehabilitative training and consider different approaches of combinatorial rehabilitative schedules. Finally, we discuss potential future strategies for designing repair and rehabilitation paradigms by introducing a "3 step model": determination of the metabolic and plastic status of the brain, pharmacological enhancement of its plastic mechanisms, and stabilization of newly formed functional connections by rehabilitative training.

Keywords: stroke, rehabilitation, Nogo-A, critical time window, plasticity, training

INTRODUCTION

The human brain works wonders to fulfill the requirements of every-day life. These unique capacities are then fully esteemed when all of a sudden even simple activities fail or become a problem: cerebral strokes leave the victims with often large psychical and physical impairments—from vision problems to aphasia and motor deficits—leading to the number one cause of adult disability worldwide with great impact on public health. In the acute phase, "time is brain"—ruptured blood vessels (hemorrhagic stroke) or aggregates of platelets and blood cells that clog cerebral blood vessels (ischemic stroke) cause acute shortage of glucose and oxygen resulting in metabolic distress and long-term neuronal cell loss. The destruction process is complex and can only be dampened in the case of the ischemic stroke by very early intervention (within 4–6 h) with thrombolysis, (Hacke et al., 2008). Currently, only about 10% of all stroke patients reach a hospital early enough or fulfill the criteria for being able to receive thrombolysis in the therapeutic time window. Prognosis and recovery then depend on the location and extent of the stroke lesion. Clinically, the most successful therapy to further enhance this recovery of function is rehabilitative training. Rehabilitation as a term "to reach and maintain optimal functioning in physical, intellectual, psychological and/or social domains" (WHO. International classification of functioning disability Health ICF. Geneva: WHO; 2001) is evidence based medicine and does not exclude a specific subgroup of patients.

Nevertheless, for many rehabilitative interventions, in particular those for long-term or chronic rehabilitation, robust data or adequately controlled studies are lacking (Quinn et al., 2009): e.g., comparisons between different training methods in current use could not show that any particular physiotherapy or stroke rehabilitation strategy is superior to another (Johansson, 2000).

Consequently optimal rehabilitation strategies can only be defined if we understand the way in which training and the rehabilitation protocol influences the neurobiology of the central nervous system with priority on the aspects of timing, kind and intensity of rehabilitative training. Measurable endpoint criteria for rehabilitative outcome are required in order to achieve two purposes: the adjustment of the ideal rehabilitative strategy to the individual patient, and the choice of the optimal therapy protocol.

In this review we focus on mechanisms of spontaneous recovery after stroke, on rehabilitative designs to enhance plasticity, on growth promoting mechanisms with an emphasis on anti-Nogo-A immunotherapy, and on the time windows of rehabilitative training and pharmacological interventions and the combination of both.

MECHANISMS OF SPONTANEOUS RECOVERY AFTER STROKE—FROM HUMAN PATIENTS TO ANIMAL MODELS

For many years people have thought that the hardware of the brain is that "hard", that once an incident such as stroke happens, brain areas and functions are lost forever. The old paradigm of the adult CNS as a stable and static structure, consisting of billions of nerve cells and circuits, has now been replaced by a much more dynamic view of the CNS which includes processes of growth, connectivity changes and areal remodeling that can occur after CNS injury or stroke and plays an important role in recovery and functional repair.

Spontaneous recovery is seen in stroke patients weeks to months after the incident. However, due to variability across subjects and across neurological domains efforts of summarizing this process with precision have been frustrating. Among the most obvious factors that contribute to the extent of spontaneous recovery are infarct size, infarct location, age and pre-stroke disability (Cramer, 2008). Most spontaneous recovery tends to occur within the first 3 months. While patients with milder deficits achieve spontaneous recovery more quickly than patients with more severe deficits, the pattern of spontaneous recovery can also differ within the same patient for different functions (Cramer, 2008).

SPONTANEOUS RECOVERY OF SENSORIMOTOR FUNCTION IN HUMANS

Motor recovery has been among the most often examined because motor impairments belong to the symptoms that are most frequently and precisely diagnosed after stroke (Gresham et al., 1998; Rathore et al., 2002; Langhorne et al., 2009). Motor impairment can be regarded as a loss or limitation of function in muscle control or movement or a limitation in mobility. It is a focus of physiotherapy or occupational therapy in terms of stroke rehabilitation (Langhorne et al., 2009). The natural history of motor recovery is considerably heterogeneous: the first voluntary movements can be seen anywhere from 6 to 33 days after a hemiplegic stroke (Twitchell, 1951). The largest improvement occurs in the first 30 days after stroke, though significant progress is still found in patients with more severe deficits up to 90 days after stroke (Wade, 1983; Duncan et al., 1992, 1994, 2005). Studies on arm disability revealed that a maximum of function is reached by 80% of the patients within 3 weeks and by 95% of patients within 9 weeks (Nakayama et al., 1994). Still significant long-term improvement is found if arm function starts to ameliorate 16 weeks after stroke onset (Broeks et al., 1999).

Insights into the underlying remodeling and re-organization processes for functional recovery in the brain after stroke can be obtained in human patients via functional neuroimaging methods and brain mapping. These data suggest that recovery of motor function after stroke leads to brain-wide modifications in neuronal activity patterns and connectivity (Rehme and Grefkes, 2013). While initially tissue function and neurophysiological responses are diminished within the injured primary neocortex, cortical function increases over time (Marshall et al., 2000; Calautti et al., 2001; Feydy et al., 2002; Grefkes and Fink, 2011). In terms of good functional outcome one of the major correlates is the degree of recovery of neurophysiological activity in the affected primary cortical areas (Cramer, 2008). In other terms: the best behavioral outcomes are associated with the greatest restoration/remodeling of brain function towards the normal state of organization (Ward et al., 2003; Zemke et al., 2003; Ward, 2004; Murphy and Corbett, 2009). This is true even if the post-stroke behavior is far from being identical to the pre-stroke motor kinematics. In particular the extent of corticospinal tract integrity is positively correlated to functional recovery as revealed by transcranial stimulation of the motor cortex (M1) and its efferents after stroke (Talelli et al., 2006). In general, if an ischemic event occurs, those areas are recruited for structural and functional modification which are either close or functionally related and connected or both. Therefore, after a small stroke, peri-infarct tissue is mainly involved that has similar function. By contrast, after a large stroke, tissue that has similar functions might be only found at more distant sites or in unaffected regions of the contralateral hemisphere, where still enough capacity for structural remodeling remains (Murphy and Corbett, 2009).

THE ROLE OF THE PREMOTOR AND CONTRALESIONAL MOTOR CORTEX

Which areas are activated and what they contribute in terms of beneficial re-organization for functional recovery is still under debate: a meta-analysis revealed that activation of premotor areas and the contralesional primary M1 are consistent findings (Rehme et al., 2012; Rehme and Grefkes, 2013). Interactions between premotor areas and the lesioned primary M1 are directly related to recovery and functional outcome. For example, Johansen-Berg et al. (2002) showed that disruption of dorsal premotor cortex activity by transcranial magnetic stimulation (TMS) over both the ipsi- and contralateral hemisphere lead to a deterioration of performance in stroke patients, but not in healthy controls (Johansen-Berg et al., 2002). The exact role of the activation of contralesional M1 is a subject to controversy: longitudinal functional MRI studies revealed enhanced neuronal activity in motor-related areas in both hemispheres after a large stroke. But then during the first 12 months post-stroke this activity returns to unilateral levels similar to those of healthy controls for those patients with good motor recovery (Ward et al., 2003). Remaining increased activity in the contralesional M1 was often associated with poor outcome. Further studies have demonstrated that inhibition of contralesional M1 activity using repetitive TMS may lead to ameliorated motor performance of the stroke-affected hand in the subacute and chronic phase (Nowak et al., 2008; Takeuchi et al., 2012). In contrast, Rehme et al. (2011) found that increases in contralesional M1 activity over the first 10 days after stroke correlate with the amount of spontaneous motor improvement in initially more impaired patients. These data suggest a supportive role for functional recovery in the early phase after stroke for the contralesional M1. In addition, disrupting contralesional M1 activity with TMS resulted in a deterioration of motor-performance of

the stroke-affected hand of stroke patients with capsula interna infarcts (Lotze et al., 2006). A clear time-, size or lesion-location- dependent influence of the contralesional M1, be it either beneficial or harmful for functional recovery, remains to be demonstrated.

CHANGES IN CORTICAL EXCITABILITY, LATERALIZED ACTIVATION AND SOMATOTOPIC RE-MAPPING

For the above described remodeling and recruitment of areas three main forms of reorganization have been described: (1) increased cortical excitability in cortical regions distant from, but connected to the stroke core; (2) reduced lateralized activation; and (3) somatotopic modifications within intact cortical regions.

Increased activity, as a first form of reaction to stroke in areas which before stroke formed a distributed network, has been described many times (Brion et al., 1989; Chollet et al., 1991). This phenomenon occurs in several cortical areas which include motor, language, attention and visual functions (Cramer, 2008). Widespread areas of cortical hyperactivity appear days after stroke and diminish within months post incident (Ward, 2004). This form of modification in cortical excitability is thought to be a result of the down-regulation of the $\alpha 1$ γ-amino butyric acid receptor subunit and a decrease in γ-amino butyric acidergic inhibition (Neumann-Haefelin et al., 1998).

The second form of reaction to stroke—reduced lateralized activation—reflects the increased activity in the contralesional hemisphere, which reduces the extent of interhemispheric balance as demonstrated in many stroke studies (Weiller et al., 1993; Seitz et al., 1998). Reduced lateralized activation is a common brain response not only seen in stroke but also in other neurological contexts such as epilepsy, traumatic brain injury and multiple sclerosis (Cramer, 2008). The exact function of this reduced laterality remains to be elucidated: it may be just a subtype of the described increased activity as described in the first form or a passive event reflecting a reduced interhemispheric inhibition resulting from the stroke. Another interpretation is that the contralesional hemisphere has to take over functions that were previously based in the ipsilesional hemisphere.

Both phenomena, increased cortical excitability and reduced laterality, are related to spontaneous functional recovery (Cramer, 2008). Both are time dependent, increasing in the early weeks after stroke and decreasing over months thereafter. This decrease is greater among stroke patients with stronger functional recovery while the persistent increased activity over both hemispheres is greatest in those patients with the poorest outcome (Ward et al., 2003; Cramer and Crafton, 2006). A relation to increased susceptibility for seizures and phantom pain is possible.

The third response to ischemic injury—somatotopic reorganization—implies that intact cortical regions—in particular within the perinfarct area—reassign their functions which they subserved before stroke and take over function, which have been affected or lost by the ischemic event. Some studies suggest that the largest degree of somatotopic reorganization is associated with very large stroke injuries (Cramer and Crafton, 2006). Such map shifts occur in primary and secondary cortical areas (Byrnes et al., 2001).

ANIMAL MODELS TO STUDY STROKE INDUCED CORTICAL RE-ORGANIZATION ON THE ANATOMICAL AND MOLECULAR LEVEL

As studies in stroke patients have limitations, animal models of stroke have been used to describe remodeling and reorganization processes on the macro and molecular level. Although spontaneous recovery in animals tends to occur earlier (depending on stroke size), imaging and mapping data show a number of analogues between recovery in animals and in humans: connectivity changes between sensorimotor cortex and deep grey matter structures after middle cerebral artery occlusion (MCAO) in rats were comparable to results in human stroke patients (van der Zijden et al., 2007). fMRI studies concentrating on the affected upper limb in rats have described a shift in laterality of activation after stroke such that early after stroke, brain activation during affected paw stimulation is mainly in the contralesional cortex, later after stroke activity shifts toward the normal pattern, that is the ipsilesional cortex (Dijkhuizen et al., 2001, 2003). Hsu and Jones (2006) found that the larger the ischemic insult the stronger the activity in the contralesional M1. In accordance with human studies van Meer et al. (2012) could show that functional recovery after MCAO in rats was correlated with the extent of preservation or restoration of the ipsilesional corticospinal tract in combination with reinstatement of interhemispheric neuronal signal synchronization and normalization of focal network organization.

New mapping methods allow describing somatotopic map shifts in animals in greater detail: a recent study using light based motor mapping in transgenic mice expressing light-sensitive channelrhodopsin-2 before and after focal ischemic lesions of the forelimb sensorimotor areas revealed decreased motor output in the infarcted area and spatial displacement of sensory and motor maps (Harrison et al., 2013). While strokes in sensory cortex caused the sensory map to move into the M1, a stoke in the M1 lead to a compensatory increase in peri-infarct cortical motor output, but did not affect the position or excitability of the sensory maps. *In vivo* 2-photon calcium or voltage sensitive dye imaging furthermore opens up new possibilities to study the reorganization of complex neuronal networks and their functional relevance for stroke recovery (Winship and Murphy, 2008; Stetter et al., 2013). Anatomically, different studies have demonstrated that map-shifts and re-mapping can be accompanied by axonal sprouting (Carmichael, 2003), and dendritic spine turnover (Brown et al., 2008, 2009, 2010). Using different tracing techniques, Starkey et al. (2012b) could show which neurons take over when functional map shifts occur: if the forelimb M1 in rats was destroyed, neurons in the hindlimb area took over to enable functional recovery of the forelimbs. This functional shift was based on sprouting of new axon branches from hindlimb corticospinal fibers into the cervical spinal cord, followed by retraction of the original lumbar projecting axon and thus a conversion of a hindlimb into a forelimb projecting neuron.

Animal studies have also provided first insights on underlying molecular changes. A unilateral infarct is associated with a number of growth related processes, in some cases bilaterally. These events include the induction of inflammatory markers, grow-promoting and inhibiting genes, cell-cycle regulatory genes and genes involved in synaptogenesis, dendritic branching and

neuronal sprouting as reviewed elsewhere (Li and Carmichael, 2006; Popa-Wagner et al., 2007).

Three major phases of stroke reaction and repair are often distinguished (Cramer and Crafton, 2006): the first epoch is the acute reaction to the injury and takes place in the initial hours when modifications become apparent in blood flow, edema, metabolism and inflammation. A second epoch is related to repair, starts in the first days post stroke and is on-going for several weeks. During this epoch spontaneous recovery is seen and endogenous repair related events reach their peak levels. The third epoch begins weeks to months after stroke when spontaneous recovery has reached a plateau and represents a stable but still modifiable chronic phase.

On the molecular level stroke induces neuronal growth-promoting genes in sequential waves post insult to initiate axonal sprouting in the peri-infarct cortex, as initially shown in a rat somato-sensory cortex (barrel field) infarct model (Carmichael et al., 2005): in the early phase immediate early genes and growth related mRNAs such as SPRR1 are induced 3–7 days after stroke. Typical growth cone constituents such as GAP43, CAP23 and MARCKS as well as the transcription factor c-Jun are expressed from day 3 onward. Subsequently, the cell adhesion molecule L1, cyclin-dependent kinase inhibitor p21 and embryonic tubulin isoform alpha1 tubulin are induced, followed by the expression of cytoskeletal reorganization genes such as SCG10 and SCLIP. This pattern of growth gene expression described is unique for axonal sprouting as a stroke response compared to expression profiles in neuronal development, peripheral or other CNS injuries (Li et al., 2010). Furthermore, in an early response to stroke (Mattson, 2008; Carmichael, 2012), several neurotrophic factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin 3 (NT-3) as well as fibroblast growth factor (FGF)-2 and insulin-like growth factor (IGF-1), epidermal growth factor (EGF) and glial cell line-derived neurotrophic factor (GDNF) are up-regulated. Each neurotrophic factor species shows a different temporal and cellular distribution pattern (Abe, 2000): while GDNF is mainly expressed by neurons, CNTF induction was predominantly observed in astroglia of the marginal region and VEDF gene expression was found in both non-neuronal and neuronal cell types after stroke.

Axonal sprouting not only requires the induction of growth-promoting programs within perinfarct neurons, but also a reduction in the growth inhibitory environment (Carmichael, 2006): axonal growth inhibition in the adult CNS is mediated through three general classes of proteins: myelin associated proteins (Nogo-A, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein), extracellular matrix proteins (e.g., chondroitin sulfate proteoglycans) and repulsive cues for growth cones known mainly from development (e.g., ephrins, semaphorins). Interestingly, messenger RNAs for the chondroitine sulfate proteoglycans aggrecan, phosphacan and versican were found to be induced later after stroke than the early and middle phase of the growth-promoting gene expression. A small number of growth inhibitory proteins including Nogo-A (Jiang et al., 2009), ephrin A5, semaphoring IIIa and neuropilin 1 are induced in the early phase, however, but down-regulation of Nogo receptor components were also seen (Li et al., 2010).

Not only a temporal expression pattern of growth promoting and inhibiting genes can be detected, but also the spatial distribution plays a role to induce the brain's self-repair processes at the right location: axonal sprouting e.g., in the peri-infarct cortex takes place in a distinct environment close to but larger than the glial scar. Thus, within the glial scar representing the wall that separates the stroke core from the surviving per-infarct tissue both, growth-promoting and growth inhibiting factors are induced while the growth-permissive and peri-infact cortex shows a reduction of the levels of growth inhibiting molecules such as chondroitin sulfate proteoglycans. In contrast, neurotrophins such as BDNF are highly up-regulated in the growth-permissive penumbra und repressed in the stroke core (Lanfranconi et al., 2011).

Taken together, the data on the time and space dependent processes of intrinsic repair mechanisms after stroke suggest a critical period or time window, in which the CNS recruits factors for plasticity that enhance functional recovery. One of the most crucial questions that has to be addressed from a clinical perspective is whether this period characterized by map shifts, fiber growth and major functional and structural changes is also the time window in which rehabilitative interventions should be initiated. We now give an overview on rehabilitative and repair strategies with an emphasis on timing, kind and intensity.

STRATEGIES TO ENHANCE PLASTICITY AFTER STROKE

GROWTH AND PLASTICITY ENHANCING TREATMENTS

Since the discovery of nerve growth factors and factors that prevent neuronal outgrowth and survival, it became a goal in experimental animal studies to apply or induce growth-promoting factors and inhibit the inhibiting ones. Several preclinical studies have examined various growth factors, hormones and cytokines with the aim to enhance motor rehabilitation—including prominent candidates such as NGF, glia (GDNF) and BDNF, IGF, erythropoietin and the granulocyte colony-stimulating factor. All have met with variable levels of success in animal models; some initial clinical studies have started (The BDNF study group (Phase III), 1999; Nagahara and Tuszynski, 2011).

In adult rats with large strokes, the administration of BDNF resulted in improved recovery rates (Schäbitz et al., 2004), while the beneficial effect of rehabilitation on the improvement of forelimb function was prevented in animals treated with a BDNF antisense oligonucleotide (Ploughman et al., 2009). The translation of these results into clinical trials remains challenging and is a matter of safety concerns: in the case of BDNF applied as a neuro-protective agent after stroke, the administration of very large quantities would be necessary as well as repeated dosing to overcome the limited amount of protein that reaches the CNS, even with transient disruption of the blood-brain barrier after stroke. The adverse effects of these high dosages have not been extensively studied in animal models (Nagahara and Tuszynski, 2011). Furthermore, the largest clinical trial of erythropoietin therapy revealed that, compared with placebo, erythropoietin administration was associated with an increased risk of mortality in patients with acute stroke (Ehrenreich et al., 2009).

Other experimental approaches to enhance the intrinsic regeneration ability of CNS axons include injecting cAMP analogs to

influence intracellular signaling pathways (Hannila and Filbin, 2008), knock down of the protein synthesis inhibitor PTEN (Liu et al., 2010) or blocking the small GTPase RhoA (Ellezam et al., 2002).

Promising results have also been gained if inhibition of neuronal plasticity and outgrowth was decreased either by: (1) digesting growth restricting ECM proteoglycans with enzymes such as chondroitinase ABC; (2) by blocking the growth inhibitory protein Nogo-A; or (3) by grafting growth permissive cells.

The bacterial enzyme chondroitinase ABC digests the glycosaminoglycan chains of the chondroitin sulfate proteoglycans (CSPGs) which are part of the extracellular matrix and usually up-regulated in astrocyctes and oligodendrocytes after CNS injury (García-Alías and Fawcett, 2012). Chondroitinase ABC treatment reduces scar formation and enhances axonal regeneration and sprouting as first shown in several studies after experimental spinal cord injury (Moon et al., 2001; Bradbury et al., 2002; Huang et al., 2006). After stroke, chondroitinase ABC administration promoted functional recovery (Hill et al., 2012; Starkey et al., 2012a). Furthermore, Soleman et al. (2012) could demonstrate that delayed chondroitinase ABC microinjections into the cervical spinal cord induce localized plasticity of the forelimb sensorimotor spinal circuitry without effects on the cortical peri-infarct region.

Inhibition of Nogo-A signaling in animal models of stroke

The well-studied protein Nogo-A, a transmembrane protein of about 1200 amino acids including a C-terminal 200 amino acid reticulon (RTN) domain, is involved in several cellular and molecular events contributing to the failure of CNS axons to sprout and reconnect after CNS injury. Function-blocking antibodies against Nogo-A, Nogo receptor (NgR1)-blocking peptides, antibodies against the Nogo receptor subunit Lingo-1, or pharmacological blockade of the signal transducer RhoA and ROCK have been administered in various laboratories in different stroke and spinal cord injury models in rodents and primates (Pernet and Schwab, 2012 for review). Enhancement of behavioral recovery in a variety of sensory-motor tasks as well as anatomical evidence of fiber growth, increased plasticity and re-organization within the cortex, brain stem and spinal cord have been reported (Zörner and Schwab, 2010 for review). Despite different approaches to interrupt Nogo-A signaling, a high degree of similarity in terms of functional recovery and hardware changes in the CNS was found among research groups and injury models. Acute intrathecal anti-Nogo-A antibody infusion over 2 weeks after stroke, with an application starting early after incident (Wiessner et al., 2003; Tsai et al., 2007), or delayed application starting 9 weeks after stroke in adult rats (Tsai et al., 2011) significantly improved forelimb function and was correlated with a significant increase of midline crossing corticospinal fibers originating in the unlesioned sensorimotor cortex. Robust sprouting of new projections from contralesional brain regions into subcortical structures as well as functional reorganization of contralateral sensorimotor areas were reported after anti-Nogo-A immunotherapy in rats (Markus et al., 2005; Cheatwood et al., 2008). Those newly sprouting cortico-efferent axons terminated in the red nucleus, pontine nuclei and spinal cord. A similar effect was found by down-regulation of the Nogo receptor NgR using adenovirus-mediated RNA interference (Wang et al., 2010) or NgR or Nogo-A/B knockout mice (Lee et al., 2004). Anti-Nogo-A immunotherapy was also associated with increases in dendritic length, complexity, and spine density, both in the lesioned and contralesional hemisphere (Papadopoulos et al., 2006). Functional MR-imaging 8 weeks after unilateral MCAO revealed adaptations in the somatosensory system of rats in the anti-Nogo-A antibody treatment group (Markus et al., 2005). Nevertheless anti-Nogo-A immunotherapy is not neuroprotective in the sense that it would reduce stroke lesion size as reported for anti-MAG immunotherapy (Irving et al., 2005). This opens the therapeutic window for anti-Nogo-A immunotherapy in the subacute and even chronic phase.

The described *in vivo* experiments represent essential preclinical tests to validate the efficiency and safety of intrathecal Nogo-A antibody administration. Three different anti-Nogo-A antibodies (IN-1, 11C7, 7B12) have proved efficient in enhancing axonal regeneration and outgrowth both *in vitro* and *in vivo*. In collaboration with Novartis Pharma, a human anti-human Nogo-A antibody has been developed and tested in extensive toxicological studies with intrathecal antibody application in rodents and primates. In a Phase I clinical trial[1] with 52 acutely injured para- and tetraplegic patients in Europe (European Multicenter Study about Spinal Cord Injury, EMSCI[2]) and Canada pharmacokinetics, safety, tolerance and dosing of intrathecal delivery of the antibody were investigated. The tolerance has been excellent without any adverse effects ascribed to the anti-Nogo-A antibody (Abel et al., 2011). A placebo-controlled Phase II clinical trial is currently in preparation. Anti-Nogo antibodies are also in clinical trials or in preparation for clinical trials for other neurological indications such as multiple sclerosis and amytrophic lateral sclerosis (ALS). For ALS GlaxoSmithKline (GSK) has also developed a humanized anti-Nogo-A antibody (GSK1223249). In a Phase I clinical trial, the intravenous injections of GSK1223249 were well tolerated by the 76 patients enrolled in the study (Pradat et al., 2011).

Several additional molecules restricting axonal growth *in vitro* have been identified including ephrins, netrins, semaphorins and oligodendrocyte myelin glycoprotein (OMgp; Schwab, 1990, 2010; Schwab et al., 1993). Their role *in vivo* after stroke has to be evaluated. How much growth and plasticity of the adult, stroke-injured CNS can be enhanced by single or combined manipulations of growth promoting or inhibitory mechanisms, and if there is a danger of chaotic growth and formation of wrong connections is currently unknown.

Finally, grafting growth permissive cells, such as bone-marrow mesenchymal cells, cord blood cells, fetal cells and embryonic cells as a form of restorative therapy have been studied in animals (Chopp and Li, 2002). E.g., cultivated bone-marrow stromal cells from donor rats were stereotactically implanted into the peri-infarct area in rats resulting in significant recovery of somatosensory behavior. In a first small study, 5/30 stroke patients who

[1]http://clinicaltrials.gov/ct2/show/NCT00406016
[2]www.emsci.org

received autologous bone-marrow mesenchymal cell transplantation showed beneficial effects in clinical stroke scores (Bang et al., 2005). Such cell-based therapies could influence endogenous neurogenesis, axonal sprouting and synaptogenesis in ischemic brain tissue (Zhang and Chopp, 2009), although their effects may be primarily immune-modulatory or neurotrophic. More detailed and systematic studies are certainly needed.

REHABILITATIVE TRAINING IN CLINICAL AND EXPERIMENTAL STUDIES

The brain, including the motor system, learns by repetition and training. Many basic mechanisms, however, are still poorly understood, and rehabilitative training is largely evidence-based medicine (European Stroke Organisation (ESO) Executive Committee; ESO Writing Committee, 2008). Nevertheless there are no generally accepted guidelines and no definite recommendations concerning the timing, kind and intensity of rehabilitative training. Clear end point data and randomized controlled clinical trials are often lacking. Furthermore, stroke recovery is a complex process that probably occurs through a combination of restoration, substitution and compensation of functions. For this reason it has been also difficult to translate results from rehabilitative studies in animals to recommendations for rehabilitative schedules in human stroke patients. A majority of clinical studies has been conducted in chronic stroke patients (> 6 months after the stroke) as recruitment of these patients was easier and baseline performance had stabilized (Krakauer et al., 2012). These circumstances lead to functional outcome measurements probably gained largely from compensatory techniques to improve skills for daily living. In contrast, animal studies had a stronger focus on enhancing impairment with more or less detailed analysis how much of the functional recovery was restoration of baseline (motor) function or compensation. Furthermore, the time courses of motor recovery differ among animal and human studies: While recovery in rodent models reaches its maximum around 4 weeks after stroke, human stroke survivors complete most of their recovery within 3 months (Dimyan and Cohen, 2011; Krakauer et al., 2012).

Early vs. delayed training

A consensus exists that the effects of early training, whereby "early" should be starting at 1–2 weeks in animals, not earlier (see below), exceed effects of delayed training in terms of functional recovery in both, animals and humans (Nudo, 2006; Murphy and Corbett, 2009; Langhorne et al., 2010; Krakauer et al., 2012). In animal studies, behavioral training after ischemic injury is most effective for restoring behavioral performance, peri-infarct neurophysiological maps and enhanced neuroanatomical changes in the ipsi- and contralesional hemisphere when introduced within the first week of injury (Nudo, 2006). In a rat MCAO stroke model it was demonstrated that functional outcome and dendritic branching patterns in the contralesional hemisphere were restricted when rehabilitative training was initiated 14 and 30 days post insult (Biernaskie et al., 2004). In another study by Hsu and Jones (2005), rats were trained in a skilled forelimb reaching tasking starting 4 or 25 days post stroke. Reaching performance was significantly enhanced in the early trained group. In a small ischemic insult in M1 in squirrel monkeys delayed training resulted in a large decrease in spared hand representation during the spontaneous recovery period that persisted following the delayed training (Barbay et al., 2006).

Concerns about initiating therapy too early following stroke arose from studies where lesion size and cell death rate were seen to be exaggerated after early excessive use of the impaired forelimb in rats while the unimpaired forelimb was casted (Kozlowski et al., 1996). One cause for increased lesion size following early excessive limb training might be NMDA-mediated excitotoxicity in the already hyperexcitable peri-infarct region (Humm et al., 1999). In closer resemblance to clinical practice were animal studies, where training or enriched rehabilitation was initiated a few days after stroke. In these cases early intervention (1–3 days post stroke) again was associated with increased cell-death but also with much improved motor performance on the long-term (Risedal et al., 1999; Farrell et al., 2001). Here, neuronal cell death may be part of a pruning effect in which non- or dysfunctional neurons are eliminated early due to a use-dependent selection. In summary, the overall consensus from animal data is that initiating rehabilitative training 5 or more days after stroke is mostly beneficial and has no adverse effects (Krakauer et al., 2012).

Constraint-induced movement therapy (CIMT), robot assisted training and electrical devices to stimulate the rehabilitation process

For human stroke patients two advanced rehabilitative approaches have proven beneficial for functional outcome: constraint-induced movement therapy (CIMT) and robot-assisted training for upper limb function (Langhorne et al., 2009; Liao et al., 2012; Mehrholz et al., 2012). Extensive preclinical studies in rodents and primates have preceded both rehabilitative strategies (Taub et al., 2002). When somatic sensation is surgically abolished from a single forelimb in a monkey, the animal avoids the usage of this forelimb in the free situation, but monkeys can be induced to use the de-afferented extremity by restricting movement of the intact limb continuously for a period of days. This concept was successfully brought into the clinics when chronic stroke patients wore a sling or cast on their less affected arm during 90% of their waking hours for 14 days (Taub et al., 1993). These patients showed a significant increase in the skill and quality of movement as measured by two laboratory tests and a much larger increase in real-world arm use over the period of these 2 weeks than the unrestricted control group. Two studies addressed the question of intensity and timing for CIMT: In the VECTORS study (Dromerick et al., 2009), 52 stroke patients were randomized at about 10 days post stroke to two levels of intensity of CIMT or standard upper extremity therapy. Intense meant 3 h of CIMT vs. 2 h of shaping therapy. After 90 days the motor outcome was worse for the more intensive CIMT group, although there had been no difference at 30 days. This result reflects the fact that too intensive CIMT can turn into an adverse situation for both the patient and the therapist. In the much larger EXCITE study (Wolf et al., 2006) patients started CIMT therapy 3–9 months post stroke and showed greater motor recovery than the usual care group. In addition Lang et al. (2013) revealed that improvements

in existing motor abilities were possible with both early (3–9 months post stroke) and delayed (15–21 months post stroke) application of CIMT. However, significant reacquisition of the ability to complete tasks was only detected with early CIMT treatment.

A number of arm and also hand training robots have been developed recently with the aim to allow very intense training without continuous, costly physiotherapy assistance. In the most modern set-ups, training devices are combined with interactive video games that can boost the motivation of the patient for the training and facility e.g., precision movements (e.g., grasping eggs and putting them into a basket). The number of well controlled and standardized outcome studies is still very limited. However, differences are discriminated between recovery of specific movements under "laboratory conditions" and functional gains for daily life activities (Mehrholz et al., 2012). Such studies are needed to exactly know the specific advantages (and potential drawbacks) of robot assisted rehabilitation in stroke (Aisen et al., 1997; Balasubramanian et al., 2010; Mehrholz et al., 2012).

Therapeutic approaches which directly stimulate the PNS or CNS electrically or by magnetic pulses may enhance neuroplasticity during poststroke rehabilitation (Dimyan and Cohen, 2011). Numerous research groups have examined the stimulation of the CNS, specifically the primary M1, by noninvasive approaches such as TMS and direct current stimulation as well as experimentally in animals by the implantation of electrodes. Several studies showed that an increase of the excitability in the stroke-affected ipsilesional M1 by electrical devices resulted in improved motor outcome (Hummel et al., 2005; Malcolm et al., 2007; Ameli et al., 2009; Koganemaru et al., 2010). The mechanisms of action of these techniques are under investigation but might involve changes in synaptic activity, gene expression and increases in neurotransmitter, receptor and neurotrophin levels (Dimyan and Cohen, 2011) or even enhanced fiber sprouting (Martin, 2012). Understanding these mechanisms may provide the basis for novel approaches using closed-loop brain machine interfaces (BMIs) that define optimal stimulation parameters from a priori developed experimental models and correctly modulate ionic currents and extracellular electric fields to provoke and guide plastic changes of the CNS (Gonzalez Andino et al., 2011).

COMBINATION OF DIFFERENT REPAIR AND REHABILITATION STRATEGIES

To maximize the effectiveness of rehabilitative therapies after stroke, it is critical to define when the brain is most responsive to sensorimotor input or extrinsic application of plasticity promoting reagents. This becomes particularly important if different rehabilitative approaches are combined.

In one of the first proof of concept studies for a critical period of heightened neuroplasticity, stroke rats were exposed to an enriched environment in combination with daily sessions of grasping training. The most significant gains in the recovery of forelimb reaching ability were achieved when rehabilitation was initiated early, i.e., 5 days after stroke as compared to 14 and 30 days after stroke. Recovery was associated with increased dendritic branching of layer V M1 neurons in the unlesioned hemisphere—a response that was not detected when rehabilitation was delayed by 30 days (Biernaskie et al., 2004).

A few recent studies in which regenerative therapies and rehabilitation have been combined have been conducted since then. These experiments suggest that designing the combination and their temporal pattern of administration are not going to be trivial (García-Alías and Fawcett, 2012; Starkey and Schwab, 2012). The different experiments have revealed a beneficial combinatorial effect, a detrimental effect, no effect at all, or an effect that depends on the relative timing of plasticity treatment and rehabilitation.

Beneficial effects were described in spinal cord injury rat models when agents against inhibitory molecules in the CNS were combined with growth promoting reagents: García-Alías et al. (2011) reported that the combination of Chondroitinase ABC with neurotrophin NT-3 and an increased expression of the NR2D subunit of the NMDA receptor resulted in better body stability and interlimb coordination compared with the single treatment groups. The behavioral data were correlated with the highest number of sprouting axons in the spinal cord and multisynaptic responses in the motor-neurons. Similar results could be found if anti-Nogo-A antibodies were combined with NT-3 and the NMDA-NR2D subunit (Schnell et al., 2011). Furthermore, the combinatorial treatment of acutely applied anti-Nogo-A antibody followed by delayed Chondroitinase ABC treatment starting 3 weeks after spinal cord injury, and forelimb grasp training starting at 4 weeks was much more effective in terms of functional recovery, sprouting and axonal regeneration than the single treatments (Rehme et al., 2011). In rats with large cortical strokes, inosine, a substance which was shown to improve fine motor control after stroke (Zai et al., 2009), augmented the effects of the Nogo receptor blocker NEP1-40 in the restoration of skilled reaching abilities in rats. Similar functional improvements were seen when inosine was combined with environmental enrichment (Zai et al., 2011).

Several recent experiments—mainly in spinal cord injury—have combined growth-promoting agents with rehabilitative training with somewhat different results: García-Alías et al. (2009) investigated whether chondroitinase-induced plasticity combined with physical rehabilitation promotes recovery of manual dexterity in rats with cervical spinal cord injury. While CSPG digestion combined with forelimb-specific rehabilitation lead to improved manual dexterity, animals treated with chondroitinase ABC in combination with environmental enrichment improved in ladder walking but performed much worse in skilled forelimb tasks than untreated control animals. In a second investigation by Maier et al. (2009) adult rats with large but incomplete cervical spinal cord injury received anti-Nogo A antibodies and simultaneous daily forced treadmill training. The simultaneous rehabilitative therapy clearly worsened the functional outcome compared with either treatment alone. When the forced treadmill training was delayed, however, for 2 weeks after the end of the antibody treatment a very good functional outcome was obtained (Marsh et al., 2011). In contrast to these results in spinal cord injured rats, combination of Nogo receptor blockade with skilled forelimb training in stroke lead to a greater degree of recovery than when either of the treatments were applied alone (Fang et al., 2010).

No additive or adverse effects were reported by Boyce et al. (2007) when neurotrophins were combined with rehabilitative training in spinal cord injured cats. Administration of pharmacological neuromodulators such as amphetamine and cholinergic agonists in combination with rehabilitative training are a matter of debate: early animal research had suggested a beneficial effect of amphetamine in recovery of motor function after stroke which could not be sufficiently reproduced in recent human and animal studies (Krakauer et al., 2012). Only for the anti-depressant fluoxetine, a serotonin-selective reuptake inhibitor, which was applied from 9 days post stroke to 3 months in a human stroke study, an impressive degree of increased motor recovery was found when combined with rehabilitative training (Chollet et al., 2011). For all these studies and their quite diverse outcomes, better knowledge of the neurobiological phenomena and mechanisms triggered by the injury, the spontaneous reaction of the nervous tissue to it, and by the different pharmacological and behavioral interventions is urgently required.

FUTURE DIRECTIONS FOR DESIGNING OPTIMAL REHABILITATION SCHEDULES

How can we better understand the neurobiology of rehabilitation? What can we learn from the above mentioned animal and clinical studies to improve current rehabilitation schedules for the best possible recovery after stroke? The presence of critical time windows for the application of growth and plasticity promoting agents and of training-dependent plasticity suggests that careful consideration of rehabilitation onset times, tailored training to the type and extent of stroke and the patient's history are required. Potential future rehabilitation schedules after stroke may therefore include the following "3 step model" (**Figure 1**):

1. Determination of the metabolic and plastic status of the brain by using state-of-the-art imaging technologies and metabolic markers
2. Enhancement of the plastic status of the brain by the application of growth and plasticity-promoting factors
3. Selection and stabilization of newly formed functional connections by rehabilitative training

One obstacle of the implementation of the optimal restorative therapies is the heterogeneity of stroke as injury location and size differ widely from one patient to another. The ability to assign the right therapy to the right patient would maximize treatment effects. Although clinical scores and a number of imaging methods exist for evaluating the state of the central nervous system and its function after stroke as reviewed elsewhere (Burke and Cramer, 2013), these approaches are often insensitive, cost intensive and have logistical difficulties. Nevertheless, neuroimaging is not only essential for the establishment of acute stroke diagnosis but can also serve as a powerful tool for the characterization of disease

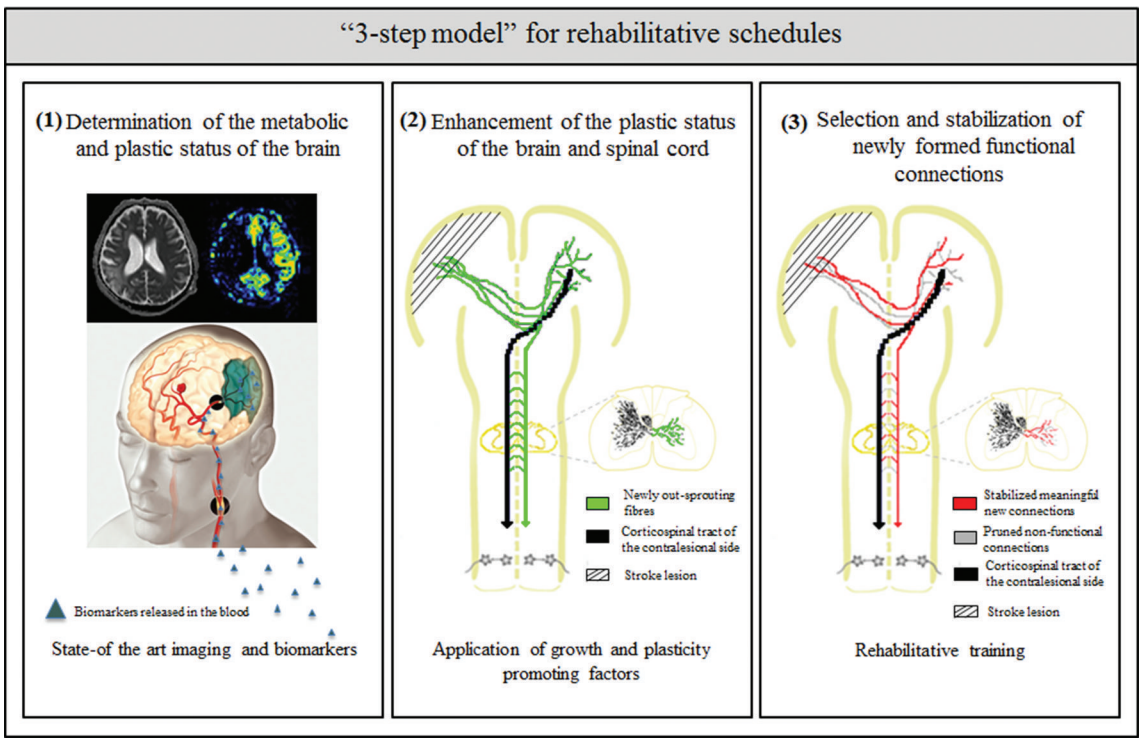

FIGURE 1 | Schematic overview of the "3 step model"—as a possible roadmap for designing future rehabilitation schedules: **(1)** determination of the metabolic and plastic status of the brain by using state- of the art imaging technologies (image taken by the Akashi Municipal Hospital, Japan) and biomarker profiles in the blood and CSF; **(2)** enhancement of intrinsic repair and plasticity mechanisms in the ispi- and contralesional hemisphere as well as the spinal cord by application of growth and plasticity-promoting factors such as anti-Nogo-A antibody or Chondroitinase ABC; and **(3)** selection and stabilization of newly formed functional connections and pruning of non-functional ones by rehabilitative training.

progression and monitoring of the response to rehabilitative interventions. Diffusion-weighted imaging (DWI) and perfusion-weighted MRI (PWI) are widely available MRI modalities that provide valuable information about the tissue characteristics of the ischemic core but also of the tissue at risk in the penumbra (Merino and Warach, 2010; Fisher and Bastan, 2012). Further work is needed to optimize the characterization of penumbra imaging for patient triage into adjusted treatment groups. In the near future we expect to learn if penumbra imaging or other early imaging features provide predictive value of critical time windows in which therapeutic interventions should be initiated or maintained and allow stratification of patients into groups for specific types of therapies.

Biomarker profiles in blood and cerebrospinal fluid (CSF) samples could bring a tremendous advance and are currently a focus of genomic and proteomic profiling studies and of systems biology in several laboratories (Stuart et al., 2010; Hemphill et al., 2011; Whiteley et al., 2012). In this regard, a biomarker or a specific combination and profile of biomarkers may not only speed up diagnosis and initiation of acute stroke treatment but may also help to classify and categorize patient groups for prediction of outcome and target the right rehabilitative approach to those stroke patients who would benefit the most.

Why do we suggest a temporal sequence of first enhancing the plastic state by growth promoting agents followed by a phase of rehabilitative training in our "3 step model"?

The current data suggest that the CNS reacts to the injury by an activation of growth and plasticity mechanisms which, however, seem to also represent a vulnerable phase in which forced activity can be harmful: this phase includes a period of GABA-mediated tonic inhibition, which may also be necessary in the first days after the stroke to limit an expansion of the infarct size (Clarkson et al., 2010), as well as homeostatic plasticity mechanisms, which ensure that neurons receive an balanced amount of synaptic input (Murphy and Corbett, 2009). Intrinsic growth and plasticity as well as exogenous enhancement of growth will lead to the formation of a large number of new connections within and between different areas of the injured CNS. In analogy to the situation in early postnatal development, many of these connections may be weak and imprecise. The functionally meaningful ones will now have to be selected and stabilized, while the malfunctional ones should be pruned, in the next, activity-dependent phase of the recovery process.

In the last step of recovery that is based mainly on rehabilitative training the spared and the new circuitry of the CNS is shaped by selection and stabilization of functional connections and pruning of the non-functional ones. Hebbian learning rules might play a crucial role in this step in the sense that Hebbian plasticity mechanisms redistribute synaptic strength to favor the wiring of pathways that are coincidently active (Murphy and Corbett, 2009). Motor learning in development is a very protracted process, requiring huge numbers of repetitions over a period of many weeks and months. Much too less is known today on the optimal time and intensity requirements for rehabilitation learning. To distinguish optimal rehabilitation schedules from less beneficial ones, strict criteria for functional outcome have to be defined that discriminate compensation and substitution from real restoration of previously impaired function. Much remains to be learned and applied in this fascinating and medically most important field of stroke rehabilitation at the interface between basic neuroscience and clinical neurology.

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SUPPLEMENTARY MATERIALS

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NEURONAL REPAIR

Asynchronous therapy restores motor control by rewiring of the rat corticospinal tract after stroke

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The brain exhibits limited capacity for spontaneous restoration of lost motor functions after stroke. Rehabilitation is the prevailing clinical approach to augment functional recovery, but the scientific basis is poorly understood. Here, we show nearly full recovery of skilled forelimb functions in rats with large strokes when a growth-promoting immunotherapy against a neurite growth–inhibitory protein was applied to boost the sprouting of new fibers, before stabilizing the newly formed circuits by intensive training. In contrast, early high-intensity training during the growth phase destroyed the effect and led to aberrant fiber patterns. Pharmacogenetic experiments identified a subset of corticospinal fibers originating in the intact half of the forebrain, side-switching in the spinal cord to newly innervate the impaired limb and restore skilled motor function.

Stroke is a major cause of severe disability in the elderly population, and recovery after large strokes is limited (*1*, *2*). Current strategies to improve long-term outcome in humans include mostly rehabilitative training and, in experimental models, electrical stimulation and pharmacological interventions (*3*). However, all of these treatment options have had only limited success thus far (*4*, *5*). Here, we show that rehabilitative training, if preceded by a nerve growth–promoting antibody therapy, almost completely restored skilled forelimb functions after cortical strokes in adult rats. Sequential application of the treatments was essential: When growth promotion by blockade of the neurite growth–inhibitory protein Nogo-A was simultaneously applied with intensive forced-use training of the forelimb during the first 2 weeks after the stroke, functional outcome was poorer compared with training, immunotherapy alone, or no treatment at all (*6*). Anatomically, Nogo-A neutralization promoted growth of corticospinal fibers from the intact forebrain motor cortex across the midline of the cervical spinal cord. In rats with simultaneous antibody treatment and training, fiber branching was abundant with anatomically aberrant terminations. In contrast, in animals trained for forelimb function subsequent to antibody treatment, axonal fibers originally terminating in the intact spinal hemicord crossed the midline and innervated the ventral motor regions of the spinal hemicord that had lost its input from the motor cortex. To prove the functional relevance of these newly grown, "side-switched" descending corticospinal tract (CST) fibers, we selectively and temporarily blocked these fibers by two different pharmacogenetic techniques, both of which suppressed the restored forelimb function. Our results demonstrate that a sequential strategy of first promoting fiber growth to enhance the low endogenous plastic potential of the brain and spinal cord, followed by rehabilitative training–induced selection and stabilization of functionally meaningful connections can lead to much higher levels of functional restoration after the formation of large brain lesions than currently obtained in conventional rehabilitation medicine.

Success of rehabilitation depends on timing

We compared four different therapy and rehabilitation schedules for promoting functional recovery of fine motor skills of forelimbs in a thrombotic stroke model in rats. Using the well-established technique of photothrombosis (*6*), we induced blood vessel blockade by multiple microthrombi, which destroyed >90% of the sensorymotor cortex of adult rats. Rats were then treated with intrathecal anti-Nogo-A or control antibody for 2 weeks (*6*, *7*). In addition, we trained rats intensely in skilled forelimb reaching (100 reaches per day), either simultaneously with antibody application (parallel groups) or during the 2 weeks after antibody treatment (sequential groups) (Fig. 1A). To avoid a training effect of testing itself, we did not reassess the sequential groups (anti-Nogo-A/sequential and control sequential) during the first 2 weeks after lesion formation. When the growth-enhancing anti-Nogo-A treatment was followed by the rehabilitative training (anti-Nogo-A/sequential group), animals improved their performance from day 16 poststroke onwards, and their skilled reaching abilities almost completely recovered [reaching 86.3 ± 2.0% of prestroke level; significantly better than all other groups; $P < 0.001$, two-way repeated measures analysis of variance (ANOVA) with post hoc Bonferroni] (Fig. 1, A and B). This group also performed best in two skilled forelimb use tasks tested at the end of the experiment

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[Montoya staircase grasping: success rate 34.1 ± 5.1% (Fig. 1C); horizontal ladder crossing: success rate 65.8 ± 3.7% (Fig. 1D)]. As animals had not been exposed to these tasks before, these results indicate a generalization of the recovery of forelimb function in the sequential training group that is transferable to nontrained motor skills.

In contrast to these results, rats receiving intensive forelimb training concurrently to the anti-Nogo-A antibody treatment (anti-Nogo-A/parallel group) performed worse than all other groups in the single-pellet grasping task (success rate 10.0 ± 5.2%) (Fig. 1, A and B). In the tasks evaluated at the end of the experiment, these animals showed either no significant improvement (Fig. 1C) or a tendency to decline over the course of trials (P = 0.1, two-way repeated measures ANOVA with post hoc Bonferroni) (Fig. 1D). The control antibody-treated groups reached low levels of recovery (35 to 40% success rate in pellet grasping) (Fig. 1, A to D), with an early training effect visible in the group trained during the first 2 poststroke weeks (control/parallel) (Fig. 1A). Final success scores did not correlate with stroke volume, as determined ex vivo from either whole-brain magnetic resonance images (r = 0.04, Spearman correlation) (Fig. 1F) or histological Nissl stains (fig. S1), suggesting no specific neuroprotective effect by any of the four therapeutic schedules. We conclude that applying a nerve fiber growth-promoting cellular therapy and rehabilitative physical training in sequence delivers greater functional recovery than when the same protocols are applied concurrently.

Rehabilitative schedules induce distinct neuronal fiber patterns

We investigated the neuroanatomical correlates of functional recovery by labeling the intact, contralesional CST, which normally innervates the spinal cord half opposite to the one that has lost its cortical input, with only few fibers crossing the spinal cord midline. Each of the four experimental groups presented a distinct rehabilitation-induced pattern of fiber sprouting in the cervical spinal cord (Fig. 2C). We counted labeled fibers originating in the cortex of the intact side opposite to the stroke and crossing the midline of the spinal cord (Fig. 2A). We also quantified their elongation and branching within the gray matter of cervical spinal cord, that is, the cord region containing the motor control circuits of the forelimb and paw (Fig. 2, A and B). The greatest number of midline-crossing fibers was seen in the anti-Nogo-A/sequential treatment group, which also had the best functional outcome. In contrast, the anti-Nogo-A/parallel group, showed extensive branching of the midline crossing corticospinal fibers

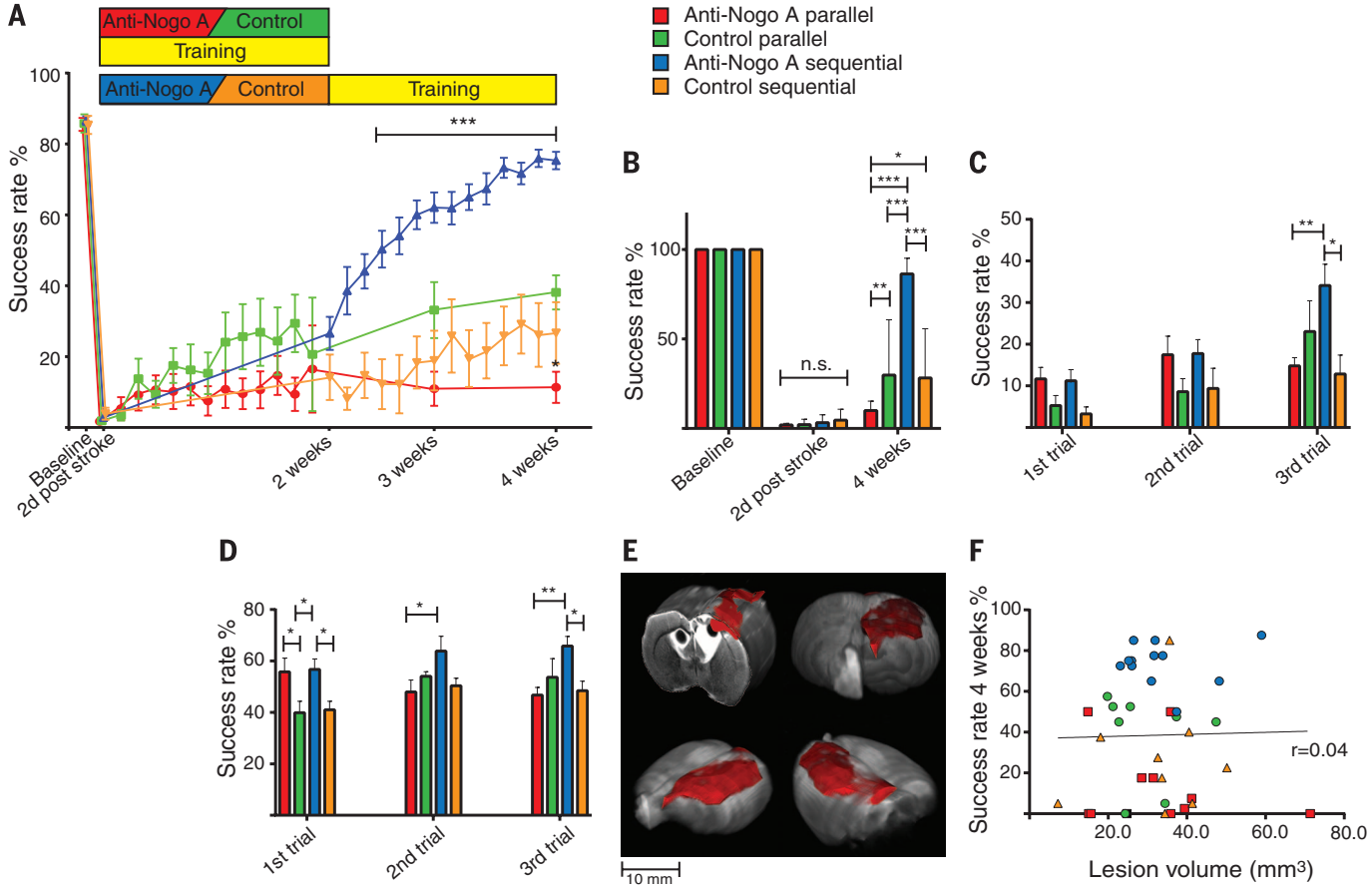

Fig. 1. Timing matters when a growth-promoting anti-Nogo-A immunotherapy is combined with training. (**A**) Success rates in the single-pellet grasping task at baseline (intact, trained), 2 days after a large, unilateral photothrombotic stroke to the sensorimotor cortex of the preferred paw, and during training and retesting sessions until 4 weeks post-insult. The anti-Nogo-A/sequential group showed significant improvement compared with all other groups, whereas the performance of the anti-Nogo-A/parallel group was significantly worse (anti-Nogo-A/parallel, n = 16; control/parallel, n = 8; anti-Nogo-A/sequential, n = 16; and control/sequential, n = 9). (**B**) Recovery rates expressed as success rates of the last testing session normalized to baseline performance (100%). n.s., not significant. (**C** and **D**) Animals in the anti-Nogo-A/sequential group also performed significantly better in grasping tasks, such as the Montoya staircase test (C) or the horizontal ladder crossing task (D), introduced after the completion of the rehabilitation schedules for three consecutive trials. In (A) to (D), data are presented as means ± SEM. Statistical evaluation was carried out with two-way ANOVA repeated measure, followed by Bonferroni post hoc test. Asterisks indicate significances: *P < 0.05, **P < 0.01, ***P < 0.001. (**E**) Representative picture of an ex vivo magnetic resonance image stack for three-dimensional stroke reconstruction. (**F**) There was no correlation between stroke volume and endpoint success rate in the single-pellet grasping task among all rehabilitation groups (r = 0.04, Spearman correlation).

(Fig. 2B) ($P < 0.05$, two-way repeated measures ANOVA with post hoc Bonferroni).

A quantitative analysis of the distribution and density of ipsilaterally projecting corticospinal fibers using pattern-recognition algorithms to analyze both single corticospinal fibers and related fiber-growth parameters (see supplementary materials and methods) confirmed overshooting fiber growth and aberrant termination patterns in the anti-Nogo-A/parallel group. In the anti-Nogo-A/sequential group, midline-crossing, sprouting corticospinal fibers displayed a radial organization with few branches and a preference for the premotor and motor spinal cord (laminae 6 to 9) (Fig. 2, D and G). In contrast, fibers in the anti-Nogo-A/parallel group appeared less organized with more than double the number of branches and a different laminar distribution including the dorsal, predominantly sensory laminae 1 to 5. We also assessed the connectivity of the ipsilaterally projecting corticospinal fibers by quantifying the density of axonal boutons recognized morphologically in the premotor interneuron lamina 7: We detected a significantly higher bouton density in the anti-Nogo-A/parallel group compared with the anti-Nogo-A/sequential group (Fig. 2H) ($P < 0.05$, Student's t test, two-tailed, unpaired). The anti-Nogo-A/parallel group showed a greater tendency of axons to grow beyond the gray matter–white matter boundary, as well as a highly aberrant growth pattern (Fig. 2, H and I). In the medio-ventral funiculus, such fibers are probably intermixed with sprouts of the small, uncrossed ipsilateral CST.

Nerve cells from the intact forebrain cortex are responsible for recovery

Our results suggest that the recovery of rat forelimb function after stroke in the anti-Nogo-A/sequential group originates from extensive and precise reinnervation of the stroke-denervated spinal hemicord by midline-crossing fibers from the intact motor cortex and CST. We tested this hypothesis in the animals of the anti-Nogo-A/sequential group, all of which showed excellent functional recovery, by using two different experimental approaches for inducible, selective, and reversible inactivation of the ipsilaterally projecting corticospinal fibers on the long and short term, respectively. For long-term blockade, we used a virus to deliver a doxycyclin-inducible tetanus toxin to temporarily inactivate the synaptic release mechanism (*8*). We injected the highly efficient retrograde gene transfer lentivector HiRet carrying enhanced tetanus neurotoxin light chain (eTeNT) with an enhanced green fluorescent protein (EGFP) downstream of a tetracycline-responsive element (TRE) into the stroke-denervated side of the cervical spinal cord at level C5-C6, and we injected the adeno-associated serotype 2.2 (AAV2) vector carrying the reverse tetracycline transactivator (rtTAV16, Tet-on) into the contralesional, intact premotor and motor cortex ($n = 6$ animals) (Fig. 3, A and B). Only cortical neurons with axons projecting to the stroke-denervated spinal

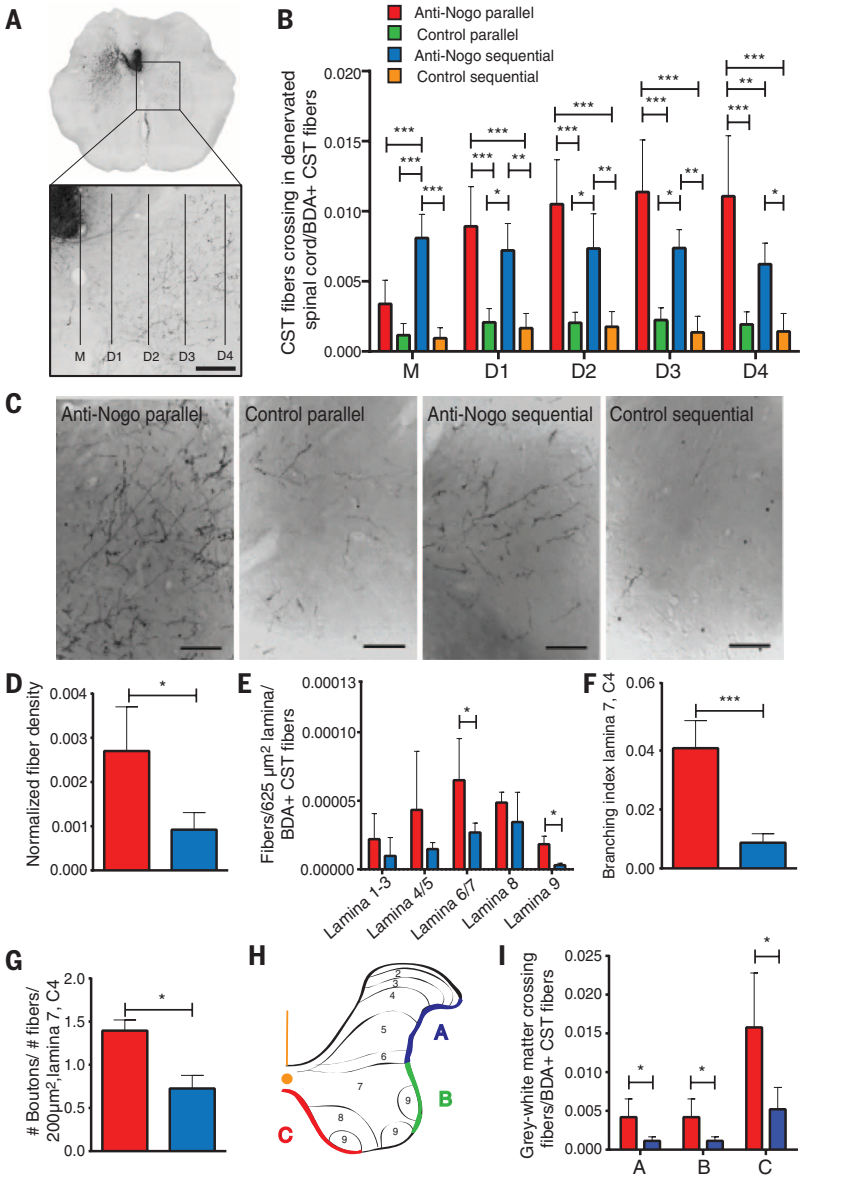

Fig. 2. Corticospinal tract sprouting depends on timing of rehabilitative training correlating with functional recovery. The four rehabilitation schedules (anti-Nogo-A/parallel, $n = 10$; control/parallel, $n = 8$; anti-Nogo-A/sequential, $n = 10$; and control/sequential, $n = 8$) differently influenced the sprouting of corticospinal fibers from the intact side of the spinal cord across the spinal cord midline (M). (**A**) Low- and high-magnification micrographs of biotinylated dextran amine (BDA)–labeled corticospinal fibers in intact spinal hemicord (left) growing into the stroke-denervated hemicord (right; inset) at spinal cord level C4. D1 to D4: Lines for intersection counts with corticospinal fibers. Scale bar, 200 µm. (**B**) Fibers crossing the midline (M) and branching in the gray matter at distances D1 to D4 were counted and normalized to the number of BDA-positive labeled fibers in the main tract. (**C**) Micrographs showing different sprouting patterns of corticospinal fibers from the ipsilateral cortex in the denervated cervical spinal cord (C4) in lamina 7 in the different treatment groups. Scale bars, 200 µm. (**D**) Combining anti-Nogo-A immunotherapy with simultaneous training (anti-Nogo-A/parallel) results in a significantly higher density of ipsilateral CST fibers in the stroke-denervated cervical spinal cord than does anti-Nogo-A/sequential treatment. (**E**) The most significant difference in fiber density between anti-Nogo-A/parallel and anti-Nogo-A/sequential animals was detected in lamina 6/7 and lamina 9 of the denervated cervical hemicord. Lamina 7 was also significant for increased fiber branching (**F**) (branching index = branches per fiber per BDA-positive fibers in the intact CST) and bouton numbers (**G**) in the anti-Nogo-A/parallel group. (**H** and **I**) Significantly more fibers cross the gray matter–white matter boundaries in the dorsolateral (labeled with "A"), the ventrolateral (label "B"), and the ventro-medial funiculus [label "C," scheme shown in (H)] in the anti-Nogo-A/parallel group. Data are presented as means ± SEM. Statistical evaluation was carried out with two-way ANOVA repeated measure, followed by Bonferroni post hoc (B) and Student's t test (two-tailed, unpaired) (D to G and I). Asterisks indicate significances: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

cord would contain both transgenes and activate tetanus toxin in response to doxycycline. We applied the same procedure in four control animals, except these animals received injections of the HiRet lentivector coding for EGFP only.

After recovery from the surgery and the reassessment of regained grasping skills, doxycycline was orally administered for 2 weeks (Fig. 3, A and C). In the experimental group grasping performance declined within a few days, reaching a very low level from day 7 after doxycycline initiation ($P < 0.05$, statistical comparison TeNT versus control group, two-way repeated measures ANOVA with post hoc Bonferroni) (Fig. 3C).When the drug administration was ceased, the lost fuction was regained within 2 weeks. All animals again showed a loss of skilled food-pellet grasping movements when oral doxycycline intake was restarted for a second time over the course of another 3 weeks (Fig. 3C). No deterioration of the poststroke recovered skilled grasping was observed in control animals (EGFP instead of eTeNT) under the same dosage of doxycycline and within the same time frame (98.1 ± 0.9% of pre-doxycycline grasping performance). As the retrogradely transported virus was EGFP-tagged, corticospinal neurons projecting to the ipsilateral cervical spinal cord segments C5 and C6 could be quantified. These neurons were concentrated in layer five of a specific region of the rostral, premotor, and primary (M1) forelimb motor cortex (3.7 ± 0.2 mm anterior to bregma). In the center of the labeled region, 41.4 ± 5.6% of Nissl-positive cells of layer five contained the transgene (Fig. 3, D and E). These results demonstrate that midline-crossing corticospinal fibers from the intact hemisphere opposite to the stroke lesion anatomically and functionally switch sides, which is crucial for the recovery of skilled forelimb movements. These fibers maintain their new function, even if they are functionally blocked for weeks. Evidently, their role cannot be compensated by other cortical or subcortical motoneuronal pathways in the injured system.

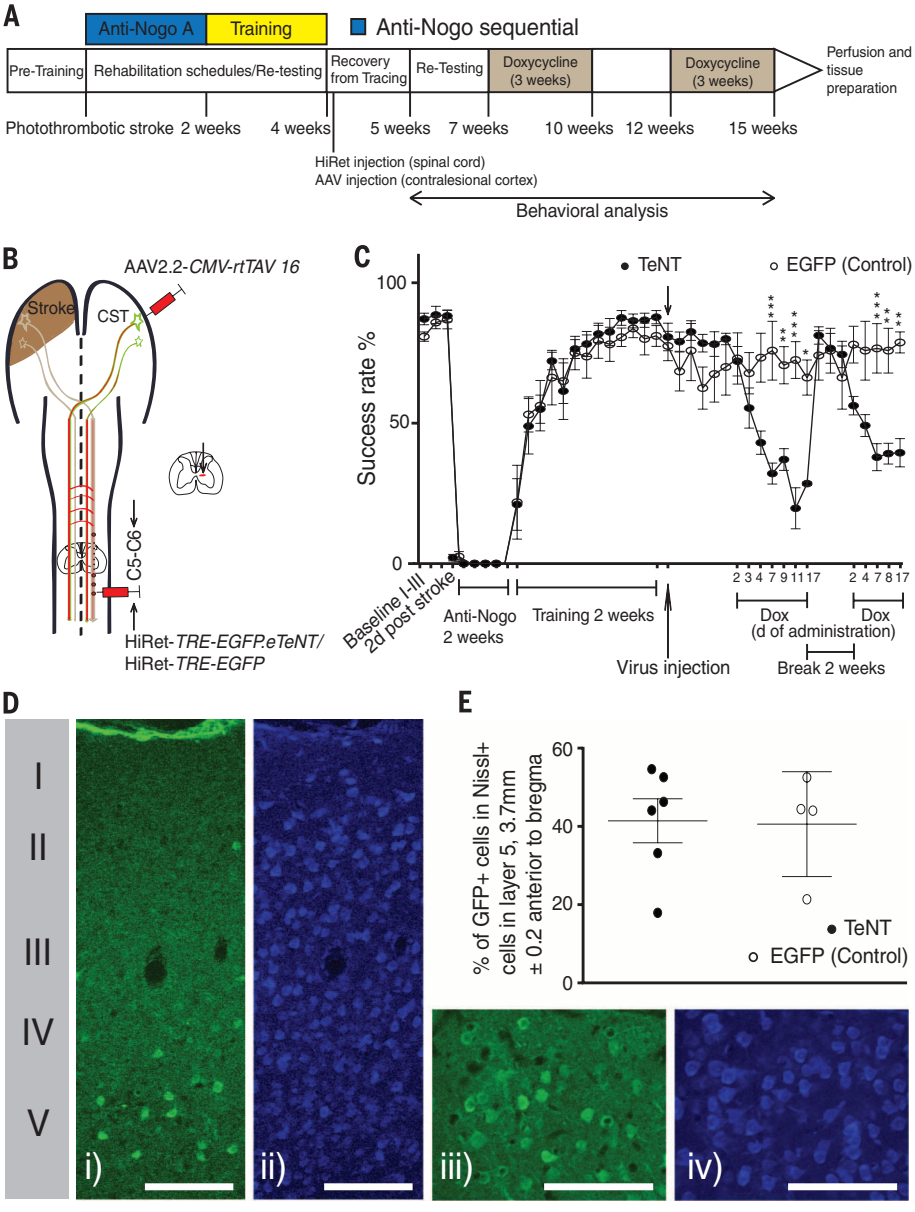

Fig. 3. Long-term reversible blockade of midline-crossing corticospinal fibers abolishes the functional recovery after stroke. (**A**) Experimental schedule. All animals had anti-Nogo-A immunotherapy followed by intensive reaching training, leading to almost full functional restoration of skilled forelimb functions. (**B**) Schematic diagram of vector injections into the contralesional motor cortex (AAV2.2-*CMV-rtTAV-16*) and stroke-denervated cervical spinal cord (HiRet-*TRE-EGFP-TeNT* or HiRet-*TRE-EGFP* as control). (**C**) Induction of tetanus toxin by doxycycline leads to strong impairment of reach and grasping movements within 7 days. The effect was fully reversible within 7 to 10 days of dox removal and could be reinduced by reapplication of dox. TeNT animals, $n = 6$; control animals, $n = 4$. (**D**) Examples of GFP-positive cells (i) in the rostral sensorimotor cortex, 3.7 mm anterior to bregma in comparison to Nissl-positive cells (ii) [scale bars for (i) and (ii), 100 μm], and zooming in to GFP-positive cells in layer five (iii) in relation to Nissl-positive cells (iv) [scale bars for (iii) and (iv), 50 μm]. Roman numerals I to V represent five out of six histological layers in the motor cortex. (**E**) Quantification of GFP-positive cells in percentage of Nissl-positive cells in the layer five motor cortex. Data are presented as means ± SEM. Statistical evaluation was carried out with two-way ANOVA repeated measure, followed by Bonferroni post hoc test. Asterisks indicate significances: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Temporally blocking rewired corticospinal fibers results in decline of regained function

For short-term reversible inactivation of the midline-crossing corticospinal fibers, we used a pharmacogenetic approach involving virus-mediated gene transfer to express engineered $G_{i/o}$-coupled DREADD receptors ("designer receptor exclusively activated by designer drug"). These receptors are only activated by the otherwise pharmacologically inert synthetic ligand clozapine-*N*-oxide (CNO), resulting in increased intracellular $G_{i/o}$-mediated signaling, which leads to membrane hyperpolarization and silencing of the infected neurons (*9*–*11*). We used only rats that had undergone the treatment schedule of growth promotion by anti-Nogo-A antibodies, followed by another 2 weeks of rehabilitative training. The virus injections started at the end of the training period (fig. S2A). To selectively manipulate the corticospinal fibers projecting from the intact, contralesional motor cortex to the denervated cervical spinal cord, we first injected an AAV2.9 vector carrying the Cre sequence into segments C5 and C6 of the stroke-denervated cervical spinal cord, followed by injections of the Cre-dependent AAV2.1 vector carrying the $G_{i/o}$-coupled DREADD (hM_4Di)

receptor into the contralesional pre- and sensorimotor cortex (n = 6) (fig. S2B). The DREADD receptor hM_4Di was tagged with mCherry, which allowed neuroanatomical confirmation that only double-infected cells expressed the hM_4Di receptor (fig. S2C): We found mCherry-positive cells concentrated in layer five of the same region of the contralesional premotor and motor cortex (3.7 ± 0.2 mm anterior to bregma), as in the tetanus toxin experiment. A portion of Nissl-positive layer five cells in this region (31.6 ± 2.4%) were positive for mCherry (Fig. 4, A and B), with very limited numbers of mCherry-positive cells outside of this area. In control animals that received injection of the Cre-dependent AAV2.1-hM_4Di vector in the contralesional cortex without AAV-Cre virus injection in the spinal cord, only 4.3 ± 0.5% of Nissl-positive cells in layer five were positive for mCherry, indicating low background noise (n = 4) (Fig. 4A).

Three weeks after virus injection, both hM_4Di/+Cre and control hM4Di/−Cre animals performed at >80% success rate in single-pellet grasping (Fig. 4C). Animals were then injected intraperitoneally with the channel-activating drug CNO. Control animals showed no change in reaching and grasping abilities over the 50 min of observation time. However, animals of the hM_4Di/+Cre group lost their grasping abilities over 10 to 30 min, with performance declining to 38.9 ± 6.0% success rate, which is significantly lower compared with the control group (Fig. 4C) (P < 0.001, two-way repeated measures ANOVA with post hoc Bonferroni). The defective movements were characterized by a specific failure to target the paw to the pellet and to close the paw around the pellet (Fig. 4D), but with little modifications in overall grasping trajectories (fig. S3). The abatement of distal motor functions was confirmed by performing discriminative classification based on a nonparametric representation (*12*) of paw posture and its change over time (P = 0.02, grasps at baseline versus after 30 min CNO, Kolmogorov-Smirnov test). This mainly distal impairment may also be due to the fact that only the cervical segments C5 and C6 were injected with the Cre virus. These functional defects were fully reversible, with performance

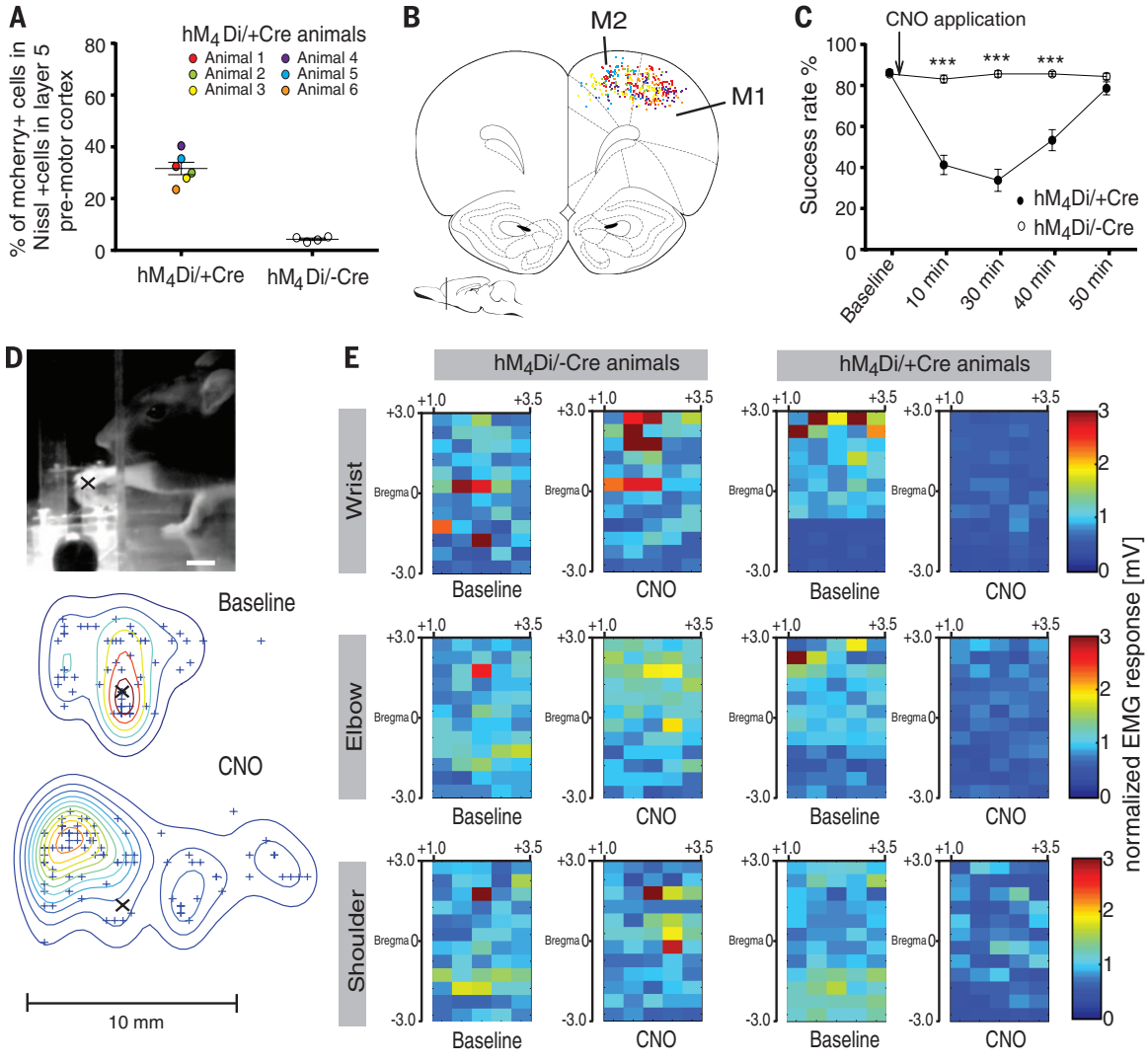

Fig. 4. Short-term reversible blockade of ipsilateral corticospinal fibers abolishes recovered grasping function and forelimb EMGs. (**A** and **B**) Quantification of mCherry-positive cells in percentage of Nissl-positive cells in layer five of contralesional premotor (M2) and motor cortex (M1), 3.7 mm anterior to bregma in the hM_4Di/+Cre group (n = 6) and the hM_4Di/−Cre control group (n = 4) (A), and the illustration of its location using Neurolucida software reconstruction (B). (**C**) Activation of the DREADD receptor in the hM_4Di/+Cre group induced a rapid and massive but fully reversible impairment of grasping performance 10 to 40 min after CNO application. Reaching success rates were unchanged in the control hM_4Di/−Cre CNO-treated group over the same time frame. (**D**) CNO application disturbed fine motor function of closing the paw around the pellet in the hM_4Di/+Cre group (P = 0.02, Kolmogorov-Smirnov test): The figure shows the spatial probability densities for the location of hand closure relative to the sugar pellet during grasping at baseline and after CNO application. "x" represents the position of the sugar pellet relative to the forelimb position during grasping. Scale bar, 10 mm. (**E**) CNO application leads to a decrease of EMG responses in the hM_4Di/+Cre group (n = 5) after ICMS of the contralesional motor cortex compared with ICMS stimulation at baseline and 30 min after CNO application in control animals (n = 3). Heat maps of the cortical stimulation grid are shown (60 stimulation points, 80 μA, +3 to −3 mm anterior-posterior and 1 to 3.5 mm medio-lateral relative to bregma). Each stimulation point is color coded with the mean value of EMG response for a muscle group (wrist, elbow, shoulder) at this stimulation point (in millivolts) normalized to the mean of all stimulation points at the baseline. Data are presented as means ± SEM. Statistical evaluation was carried out with two-way ANOVA repeated measure, followed by Bonferroni post hoc test. Asterisks indicate significances: ***P < 0.001.

returning to preinjection levels ~40 to 50 min after the CNO injection (Fig. 4, C and D).

Pharmacogenetic inhibition of regained EMG activity

We confirmed the CNO-specific blockade of neuronal firing of ipsilaterally projecting, partially midline-crossing corticospinal fibers of the intact, contralesional motor cortex by electrophysiology using intracortical microstimulation (ICMS) at the end of the behavioral testing: We used a 5-by-12–point stimulation grid (positioned at +3 to −3 mm anterio-posterior and 1 to 3.5 mm mediolateral relative to bregma) (fig. S4A) and electromyogram (EMG) recordings of wrist, elbow, and shoulder muscles of the impaired paw as readouts (fig. S4B). In all animals, each cortical position within the exploration grid was stimulated twice, first as baseline stimulation, then again 30 min after CNO injection. In all hM_4Di/−Cre control animals, the EMG responses at baseline and 30 min after CNO injection were not significantly different for the 60 stimulation points [for wrist, 95% confidence interval between baseline and 30 min CNO was for animal one: 0 (0 to 0.001), animal two: 0 (0 to 0), animal three: 0 (0 to 0.001); one-sample Wilcoxon signed rank test] (Fig. 4E and fig. S4C). In contrast, in four out of five hM_4Di/+Cre animals, the ICMS-evoked EMG responses significantly decreased 30 min after CNO injection compared with baseline [for wrist, 95% confidence interval was for animal one: −0.002 (−0.002 to −0.001), animal two: −0.004 (−0.005 to −0.003), animal three: −0.002 (−0.002 to −0.002), animal four: 0 (0 to 0.001), animal five: −0.012 (−0.016 to −0.009); one-sample Wilcoxon signed rank test] (Fig. 4E and fig. S4D). Calculating the mean EMG response for every stimulation point in hM_4Di/+Cre animals versus hM_4Di/−Cre controls revealed a significant decline of EMG responses in wrist and elbow muscles in the hM_4Di/+Cre group 30 min after CNO application compared with controls ($P < 0.05$, Mann-Whitney test) (Fig. 4E). No significant abatement of EMG responses occurred in shoulder muscles of hM_4Di/+Cre animals ($P = 0.4$, Mann-Whitney test) (Fig. 4E). CNO application resulted in the largest difference in EMG responses of wrist recordings when premotor and rostral forelimb areas of the contralesional motor cortex were stimulated in the hM_4Di/+Cre group compared with controls (fig. S2D). These areas also expressed the highest concentration of mCherry-expressing cells (Fig. 4A).

Discussion

Our study shows that in a rat model of large forebrain cortex strokes, timing of rehabilitative training relative to timing of a nerve fiber growth-promoting therapy affects the recovery of lost motor function and the pattern of fiber sprouting. When rats received their first anti-Nogo-A immunotherapy followed by 2 weeks of specific, intense rehabilitative training, forelimb function was almost fully restored (Fig. 1, A and B), indicating a far more extensive recovery rate relative to stroke size than previously obtained by training (*13*–*15*) or growth-promoting therapy alone (*6*, *16*). Not only did these animals outperform the other rehabilitation groups in the single-pellet grasping task, but they were also able to better transfer their regained skills to novel forelimb tasks (*15*). The behavioral recovery was associated with crossing of CST fibers from the lesion-spared, intact motor cortex to the stroke-denervated side of the spinal cord. This observation is supported by various stroke and spinal cord injury models (*15*–*19*) that relate midline-crossing corticospinal fibers to functional outcome. Our analysis shows that not only the quantity of newly out-sprouting corticospinal fibers is relevant but also their termination pattern: Intensive training, when applied too early, induced hyperinnervation and aberrant growth even beyond the gray matter–white matter boundary and into dorsal sensory laminae. Such widespread sprouting may result in wrong circuit connectivity involving cervical interneurons, V2a propriospinal neurons, and motoneurons, thus impairing grasping function, for example, by coactivation of agonistic and antagonistic muscles (*20*, *21*).

Our data suggest the presence of critical time windows during which the brain is most responsive to the application of growth-promoting agents and to training-dependent plasticity. A correct, timed sequence of interventions is required to maximize the effectiveness of rehabilitative therapies after stroke. In a first step, suppression of the action of the endogenous growth-inhibitory factor Nogo-A by immunotherapy may diminish constraints on lesion-induced structural plasticity through mechanisms such as neurotrophic factors, modified electrical properties of motoneurons, alteration in neuronal energy balance (*22*), and recruitment of new circuits leading to hyperexcitability and prolonged responses to external stimuli (*1*). In analogy to development, many of these newly formed connections may be weak and imprecise. Training in a second step may then help to shape the spared and new circuits by selection and stabilization of functional connections and pruning of the nonfunctional ones. This second step might involve Hebbian learning rules, in the sense that Hebbian plasticity redistributes synaptic strength to favor functionally relevant pathways that are coincidently active (*1*). The high degree of recovery of important, cortically controlled motor functions in rats with large ischemic strokes, as demonstrated here, points to a possible avenue to explore growth-inhibitor blockade in combination with rehabilitative training as a treatment strategy for humans with motor cortex stroke. Antibodies against Nogo-A are currently used in clinical trials in humans for amyotrophic lateral sclerosis, multiple sclerosis, and spinal cord injury (www.clinicaltrials.gov). Careful consideration of rehabilitation onset times—particularly with regard to windows of sprouting and circuit plasticity, but also vulnerability of the injured brain—and tailored training adapted to the type and extent of stroke and the patient's history will be essential for future approaches in the clinic (*2*, *23*).

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/344/6189/1250/suppl/DC1
Materials and Methods
Fig. S1 to S4
References (*24*–*34*)

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